UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

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CERTIFICAN

Que Doña Sara Ares Carrasco, Licenciada en Biología por la Universidad de Salamanca, ha realizado bajo su dirección el trabajo titulado "Mecanismos moleculares implicados en el daño miocárdico inducido por diabetes. Efecto del bloqueo de los receptores de aldosterona." que presenta como Tesis Doctoral para alcanzar el grado de Doctora por la Universidad Autónoma de Madrid.

Y para que conste, firmamos la presente en Madrid a 28 de Mayo del 2013

Los directores de tesis

Dr. José Tuñón Fernández

Dr. Óscar Lorenzo González

No man-made structure is designed like a heart. Considering the highly sophisticated engineering evidenced in the heart, it is no surprising that our understanding of it comes so slowly.

(Daniel D. Streeter Jr.)

"Tal y como yo lo veo, diez mil dificultades no hacen una sola duda; dificultad y duda son cosas heterogéneas."

(Beato John Henry Newman)

A mi esposo,

a mis hermanos,

a mis padres.

Me he resistido a escribir esta sección hasta que no supiera con absoluta certeza que esta tesis iba a ser publicada. Tenía miedo de que nunca viese la luz pero finalmente aquí estamos y tengo que dar gracias a infinidad de personas. Durante todo este tiempo he ido agradeciendo a todo el mundo (José Prieto ;)) su ayuda, me gusta decir las cosas en el momento porque el mañana no nos pertenece. Pero también voy a aprovechar para hacerlo aquí. Perdonad mi falta de elocuencia.

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Quiero dar gracias a todos mis queridos compañeros, y, más que eso, amigos que han estado conmigo toda esta etapa sin defraudarme nunca. Os admiro profundamente, sois un regalo. Siento haberos dado tanto la brasa con Zamora y con mis continuos cánticos. Uno por uno, a mi Concept por su bravura y su fuerza, nadie podrá contigo, muchas gracias por meterme presión tantas veces. Gracias por los momentos en New York, nos echamos unas risas. A Bea, porque compartimos muchísimas cosas, me siento muy unida a ti. Espero poder devolverte algún día todo lo que me has ayudado. A Álvaro, por todos sus gestos de cariño conmigo que han sido muchos. Ya os he dicho que os admiro, pero lo quiero repetir. Gracias a Alberto, por escucharme y por decirme siempre lo que piensa, por hablarme siempre claro, porque nos entendemos. A Carlos, qué discreción la tuya, qué templanza, porque siempre estás ahí como si nada. A Vir, por su meticulosidad y su cariño. Estamos aquí para lo que necesites. Gracias a Julio, me encanta hablar contigo y estar a tu lado. Recuerdo nuestras conversaciones en la campana en las que terminaba hablando de mi familia y alguna que otra lagrimita caía. Soy un poco (mucho) de lágrima fácil. Gracias a los que conozco "por el trabajo" pero que nunca o poco he trabajado con ellos pero que habéis sido fundamentales, sobre todo en los últimos tiempos. A Jon, cómo me encantaba cantar cuando te veía Forever Jooooon! (parafraseando a Bob Dylan). Por entonces cantaba bastante, jajaja. A Elsa, a Van, a Eva y a Cris. Qué cracks! Sois lo mejor que hay por ahí. Gracias por vuestras palabras, por vuestra alegría y sinceridad, son grandes virtudes. Nos vemos de cañas hablando de la vida. A Javi por todas sus preguntas de las que me beneficio secretamente (se acabó el secreto) ☺.

Infinitas gracias, que las dos sabemos quién y en qué medida son devueltas, a María por hacer lo que yo no podía hacer. Te agradezco tu disposición y tu ayuda. Y que tengas un cuartito en el que podamos hablar ;). 100 por 1.

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Te amo.

Resumen

La *diabetes mellitus* (DM) es considerada una pandemia mundial. En 2013, 347 millones de personas padecían diabetes. La Diabetes tipo I (DM1) o tipo II (DM2) induce un efecto deletéreo directo sobre el corazón que además podría enfatizarse por la frecuente coexistencia de hipertensión. En un modelo experimental de DM1 crónica observamos un aumento de fibrosis cardiaca intersticial y perivascular, y expresión de factores pro-fibróticos, TGF-β, CTGF y proteínas de matriz extracelular. Además detectamos un aumento de apoptosis y sobrexpresión de moléculas pro-apoptóticas FasL, Fas, Bax y caspasa-3, así como una disminución de proteína anti-apoptótica Bcl2. En ratas donde la DM1 concurrió con la hipertensión se observó además un incremento de la activación de NF-κB, del infiltrado inflamatorio y de la expresión de mediadores pro-inflamatorios como IL-1, TNF-α, MCP-1, VCAM-1, angiotensinógeno y agentes oxidantes, ausentes en DM1. En este sentido el proceso inflamatorio del corazón DM1 en estos estadios podría estar atenuado por la expresión local de moléculas anti-inflamatorias y anti-oxidantes como IL-10, catalasa y HO-1. Sin embargo, en un modelo agudo de DM1 e hipertensión, observamos que ambas patologías por separado presentaron activación de los procesos de fibrosis, apoptosis e inflamación.

Posteriormente, mediante proteómica, se observó que el miocardio DM1 crónico presentaba 24 proteínas cuya expresión estaba alterada. Algo más del doble encontramos en los corazones hipertensos. El miocardio DM1 poseía sobreexpresión de proteínas pro-apoptóticas y del citoesqueleto, como ANXA5 y LDB3, junto con disminución de proteínas anti-apoptóticas y enzimas mitocondriales, como HSPD1 y ACO2). En el miocardio hipertenso estas alteraciones estaban enfatizadas mostrando, además, un descenso en la expresión de enzimas de la β-oxidación, como ACSL1. La coexistencia de DM1 e hipertensión indujo modificaciones adicionales en enzimas de la cadena transportadora de electrones como CYC1 y CKMT2. Los listados proteicos fueron utilizados como inputs en programas bioinformáticos de predicción de mecanismos celulares y observamos la potencial implicación de receptores nucleares de ácidos grasos (PPAR) y proteínas prohipertróficas tanto en DM1 como en hipertensión. Así, confirmamos la activación de PPARα en corazones DM1 e hipertensos, y en cardiomiocitos en cultivo sometidos a condiciones de hiperglicemia y/o pro-hipertensión. Además la activación exógena de PPARα redujo la hipertrofia y la expresión de ANXA5 en estas células. Así, PPARα podría encontrarse sobrexpresado en los corazones DM1 e hipertensos para compensar la respuesta hipertrófica. Finalmente, estudiamos los mecanismos moleculares implicados en la respuesta apoptótica del corazón de un modelo de DM2-obesidad. El corazón de ratas Zucker (ZDF), exhibió disfunción cardiaca y aumento del sistema pro-apoptótico Fas. Interesantemente el bloqueo del receptor de mineralocorticoide redujo la apoptosis y expresión de proteínas del sistema Fas. Sin embargo, en cardiomiocitos en cultivo, el exceso de ácido graso indujo apoptosis mediante mecanismos independientes de Fas.

Summary

Diabetes mellitus is considered as a world pandemic disease. In 2012, 347 million people suffered from diabetes. In humans, heart injury may be directly induced by Diabetes mellitus type 1 (DM1) and type 2 (DM2) and enhanced by coexistence of hypertension. In a long-term DM1 animal model, we observed an increase in cardiac interstitial and perivascular fibrosis and the expression of fibrotic factors, such as TGF-β, CTGF and matrix proteins. In addition, chronic DM1 myocardium showed an increase in apoptosis and pro-apoptotic molecules FasL, Fas, Bax, and cleaved caspase-3 were also augmented. Bcl2 expression, anti-apoptotic protein, was decreased. When DM1 was associated with hypertension, hearts revealed NF-κB activation, increased inflammatory cell infiltrate, and expression of inflammatory mediators like IL-1, TNF-α, MCP-1, VCAM-1, angiotensinogen and oxidants, that were absent in long-term DM1. In chronic DM1, an inflammatory process was possibly attenuated due to local expression of anti-inflammatory and anti-oxidants molecules such as IL-10, catalase and HO-1. Interestingly, both acute DM1 and hypertensive hearts showed fibrotic, apoptotic and inflammatory processes activation.

Proteomics revealed that long-term DM1, hypertensive and hypertensive/DM1 myocardia presented 24, 53 and 53 altered proteins, respectively. DM1 myocardium showed over-expression of apoptotic and cytoskeleton proteins (ANXA5 and LDB3), and down-regulation of anti-apoptotic and mitochondrial enzymes (HSPD1 and ACO2). In hypertensive hearts these changes were exacerbated and free fatty-acid β-oxidation enzymes (such as ACSL1) were additionally decreased. Furthermore, DM1/hypertensive myocardium exhibited a misbalance of specific pro-hypertrophic, anti-apoptotic and mitochondrial ATP-carrier factors (ex. CYC1 and CKMT2). Differential proteins were clustered into different biological pathways using bioinformatics in order to get potential implicated factors and mediators. Fatty acid nuclear receptors [43] and hypertrophic proteins were tagged as principal agents. In this regard, DM1 and hypertensive hearts showed PPARα activation and PPARα reduced hypertrophy and pro-hypertrophic factors such as ANXA5 in high-glucose and AngII stimulated cardiomyocytes. Thus, activation of PPARα could reflect a compensatory response to the metabolic-shifted, apoptotic and hypertrophic status of the hypertensive-diabetic cardiomyopathy. Lastly, we studied the molecular mechanisms, specifically apoptosis, which take place in DM2 myocardium. In the heart of ZDF rats we observed an enhancement of the Fas pro-apoptotic system as well as cardiac dysfunction. Additionally, we studied the role of eplerenone in cardiac apoptosis associated to experimental obese/DM2. DM2 hearts showed cardiac apoptosis, Fas-caspase-8-caspase-3 pathway activation and cardiac dysfunction. Treatment with eplerenon, an aldosterone receptor blocker, decreased apoptosis and the expression of Fas system components. Interestingly, in cultured cardiomyocytes, high-concentrations of palmitate promoted apoptosis by Fas-pathway independent mechanisms.

ÍNDICE

ABREVIATURAS Y ACRÓNIMOS

I. ABREVIATURAS Y ACRÓNIMOS

1. ABREVIATURAS

AngII	Angiotensina II
ANXA5	Anexina 5
β-MHC	Cadena pesada de la miosina □
CKMT2	Creatina quinasa mitocondrial 2
CoA	Coenzima A
CS	Citrato sintasa
CT-1	Cardiotrofina 1
DES	Desmina
DM	Diabetes mellitus
ENO-1	Enolasa 1
FE	Fracción de eyección
GLUT-4	Transportador de glucosa 4
FHL2	Proteína 2 de 4 dominios y medio LIM
HADHA	Proteína trifuncional mitocondrial subunidad alfa
HO-1	Hemoxigenasa 1
IL	Interleuquina
IR	Receptor de insulina
IMC	Índice de masa corporal
MCD	Miocardiopatía diabética
MEC	Matriz extracelular
MYL2	Cadena ligera de la miosina 2
MYOZ2	Myozenina 2
RAGE	Receptor de AGE
SOD	Superóxido dismutasa
VI	Ventrículo izquierdo

2. ACRÓNIMOS

ACS	Acetil-CoA sintasa
ACADl	Acil-CoA deshidrogenasa, cadena larga
ACADm	Acil-CoA deshidrogenasa, cadena media
ACADS	Acil-CoA deshidrogenasa, cadena corta
ACSF2	Acil-CoA sintasa, miembro 2 de la familia
ACSL1	Acil-CoA sintasa, miembro 1, cadena larga
ACTC1	Alfa-actina cardiaca
AGEs	Productos finales de glicosilación avanzada
ANP	Péptido natriurético atrial
AP-1	Proteína activadora 1
APAF-1	Factor activador de la proteasa apoptótica 1
ARHGAP1	Proteína activadora de GTPasa Rho
AT-1	Receptor tipo 1 de angiotensina II
ATP	Adenosina 5'-trifosfato
Bax	Proteína X asociada a Bcl-2
Bcl-2	Gen del linfoma de células B
CBP/p300	Proteína de unión a CREB
CPT-1/CPT1B	Carnitina palmitoiltransferasa 1
CTGF	Factor de crecimiento de tejido conectivo
CV	Cardiovascular
CYC1	Proteína de citocromo C1
DAG	Diacilglicerol
DIGE	Electroforesis diferencial en gel
ECA	Enzima convertidora de angiotensina
EMPHASIS-HF	Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure
EPHESUS	Eplerenone Post-AMI Heart Failure. Efficacy and Survival Study
ET-1	Receptor de endotelina 1
FABP	Proteína de unión a ácidos grasos
FADD	Factor asociado a Fas vía dominio de muerte
FasL	Ligando de Fas

FAT/CD36	Translocasa de ácidos grasos
FATP	Proteína transportadora de ácidos grasos
FGF	Factor de crecimiento de fibroblastos
FLICE	Enzima convertidora de interleuquina-1b similar a FADD
HNF-4α	Factor nuclear de hepatocito 4α
HSP	*Heat shock protein*
ICAM	Molécula de adhesión intercelular 1
IECA	Inhibidor de la enzima conertidora de angiotensina
IGF	Factor de crecimiento similar a insulina
LDB3	Dominio de unión a LIM 3
MAP p38 quinasa	Proteína quinasa p38 activada por mitógeno
MCP-1	Proteína quimioatractante de monocitos 1
MCPT	Carnitina palmitoiltransferasa específica de músculo 1
MECR	Trans-2-enoil-CoA reductasa mitocondrial
MEF2	Factor potenciador específico de miocito 2
MMP	Metaloproteinasas
NADPH	Nicotinamida adenina dinucleótido fosfato reducida
NEFA	Ácidos grasos no esterificados
NF-κB	Factor nuclear kappa B
NOS-1	Sintasa 1 de óxido nítrico
PBP/TRAP220	Proteína de unión a PPAR
PDH	Piruvato deshidrogenasa
PDHX	Componente X de PDH
PDIA3	Proteína isomerasa disulfuro A3
PDK4	Piruvato deshidrogenasa quinasa 4
PGC-1	Coactivador de PPARγ tipo 1
PKC	Proteína quinasa C
PKM	Piruvato quinasa muscular
PPAR	Receptores activados por proliferadores de peroxisomas
p-Smads	Smad fosforilada
RALES	Randomized Aldactone Evaluation Study
ROS	Especies reactivas de oxígeno.
RXR	Receptor retinoico X

SAKP	Proteína quinasa asociada a estrés
SAPK	Protein-quinasa asociada a estrés
SCR-1	Coactivador del receptor de esteroides 1
SHR	Ratas espontáneamente hipertensas
SK1	Esfingosina quinasa
SKA	Alfa-actina esquelética
SRAA	Sistema renina-angiotensina-aldosterona
SRC1	Co-activador 1 del receptor esteroide
TCA	Ciclo de los ácidos tricarboxílicos
TGF-β	Factor de crecimiento transformante β
TIMP	Proteína inhibidora de metaloproteasa
TNF-α	Factor de necrosis tumoral -α
TRADD	Proteína asociada al dominio de muerte del receptor del factor de necrosis tumoral
TRAP1	Proteína asociada al receptor TNF 1
TUNEL	Detección de UTP terminal por transferasa deoxinucleotidil terminal
UCP3	Proteína de desacoplamiento 3
UFPR	Respuesta a proteínas desplegadas
VCAM	Molécula de adhesión de células vasculares 1
ZDF	Zucker Diabetic Fatty rats

INTRODUCCIÓN

II.INTRODUCCIÓN

1. *Diabetes mellitus*

La *Diabetes mellitus* (DM) se define como un conjunto de síndromes iniciados por la aparición de hiperglicemia provocada por defectos en la secreción de insulina, disminución de la acción de la insulina o combinación de ambos mecanismos[9]. Varios procesos patogénicos están implicados en el desarrollo de esta enfermedad distinguiéndose principalmente dos tipos de DM. La *diabetes mellitus* tipo-1 (DM1), diabetes juvenil o diabetes insulinodependiente es aquella en la que se produce una destrucción de las células β-pancreáticas con la consiguiente deficiencia en insulina y asociada hiperglicemia, y cuyo origen puede ser autoinmune o idiopático. La *diabetes mellitus* tipo-2 (DM2) o diabetes no insulino-dependiente, definida como un conjunto heterogéneo de alteraciones celulares originadas por polimutaciones genéticas y que dan lugar a resistencia a insulina, agotamiento en su secreción, y aumento de los niveles glucosa en sangre[2].

1.1. PREVALENCIA

La Organización Mundial de la Salud ha pronosticado que en el año 2030 la DM afectará a màs de 400 millones de personas, lo que supone un aumento de un 114% con respecto a las cifras publicadas en 2000[92]. Actualmente existen unos 347 millones de enfermos en todo el mundo (Fig. 1), de los cuales más del 90% son pacientes con DM2[186]. Sin embargo, las cifras globales de prevalencia en adultos oscilan entre el 4% y el 7%, y muchos de ellos no han sido aún diagnosticados. La DM1 supone un 10% del total de diabéticos y, al igual que la DM2, su prevalencia está en aumento. Además, la Asociación Americana de Diabetes estima que los costes nacionales asociados a la DM en los EE.UU. para el año 2002 fueron de 132 mil millones de dólares y se prevee un aumento de unos 60 millones más en el 2020[80].

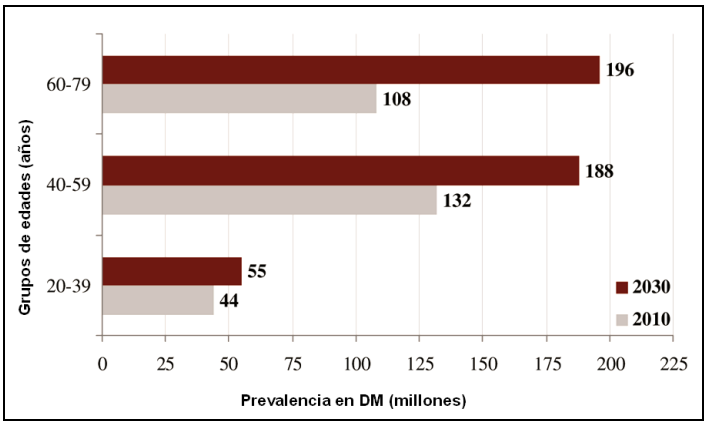

Figura 1. Prevalencia global en los diferentes grupos de edad para los años 2010 y 2030[71].

Interesantemente, la mayoría de pacientes con DM fallece por causas cardiovasculares, Se estima que, en 2012, 204 millones de personas fallecieron como consecuencia de complicaciones cardiovasculares. Así, la DM se asocia con una menor esperanza de vida, y una mayor morbilidad y disminución de la calidad de vida. Por áreas, sorprendentemente Asia, y Europa son las zonas con mayor número de víctimas y en mujeres ocurren más fallecimientos que en hombres (Fig. 2).

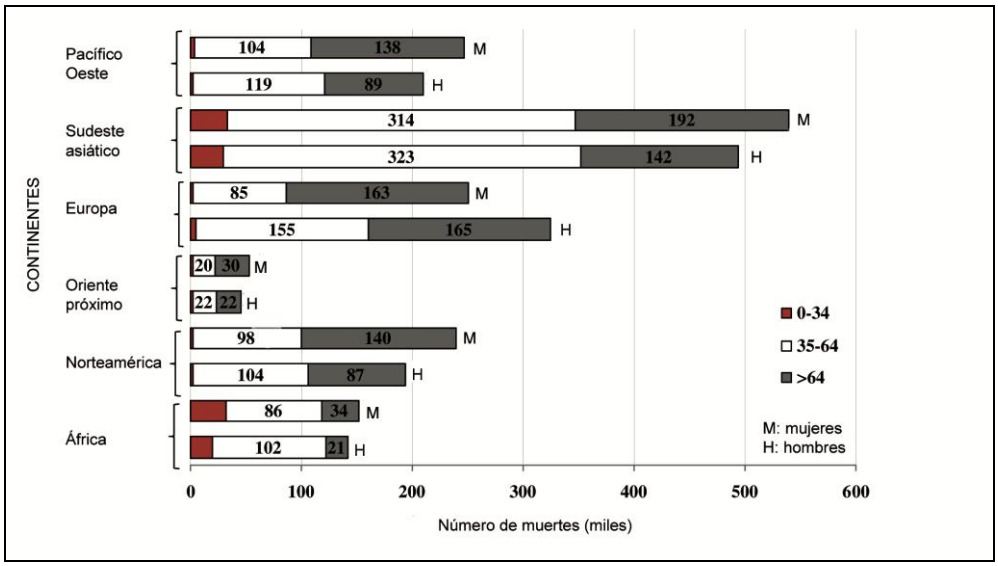

Figura 2. Número de fallecimientos (en miles) atribuidos a diabetes y clasificado por edad y género[71].

1.2. COMORBILIDADES EN DIABETES

Comorbilidad viene definida como transtorno que acompaña a una enfermedad primaria implicando la existencia de dos o más patologías médicas no relacionadas. La comorbilidad más frecuente en DM es la hipertensión. De hecho, más del 75% de adultos con DM también presentan hipertensión[33]. Hipertensión y DM son condiciones entrelazadas que comparten un significativo solapamiento en los factores de riesgo subyacentes (incluyendo determinantes étnicos, familiares y estilo de vida) y complicaciones. Estas complicaciones incluyen daños macro- y microvasculares. Estas dos patologías comparten similares cambios en la ultraestructura y función cardiaca, y en la expresión génica. Evidencias en modelos animales[49] y humanos[48,62] revelan que sus efectos son independientes y sinérgicos. La predisposición a la DM e hipertensión parece tener un origen poligénico, lo que perjudica la posible aproximación de la terapia génica para el control y prevención de estas enfermedades[131,117]. Por otro lado, los factores compartidos relacionados con el estilo de vida proporcionan la oportunidad de un primer enfoque del manejo de la enfermedad no farmacológico haciendo especial incapié en el control del peso corporal, actividad física y cambios en la dieta[105].

Otra comorbilidad es la obesidad. El riesgo de presentar DM[179], concretamente DM2, aumenta progresivamente a medida que lo hace el índice de masa corporal (IMC)[6]. El 80% de los pacientes con DM2 tienen sobrepeso o son obesos. El 18% de los obesos con IMC>40kg/m2 presentan DM2. La reducción del 5-10% del peso reduce el riesgo de sufrir DM2 en un 30% y mejora las concentraciones plasmáticas de glucosa e insulina[6]. Los mecanismos a través de los cuales la obesidad y, concretamente, el aumento de grasa visceral produce insulinoresistencia se explican por el aumento en la secreción, por parte del tejido adiposo, de algunos péptidos como la leptina, la adiponectina y diversas citoquinas [factor de necrosis tumoral (TNFα), interleuquina 6 (IL-6)][163].

1.3. EXPRESIÓN CLÍNICA: SÍNTOMAS Y COMPLICACIONES

Los síntomas de la DM1 incluyen excreción excesiva de orina (poliuria), sed (polidipsia), aumento del apetito (polifagia), pérdida de peso, cambios en la visión y fatiga. En cuanto a la DM2, los síntomas pueden ser similares a los de la DM1, pero menos acentuados[2]. Como resultado, la DM2 puede ser diagnosticada varios años después del inicio, una vez que ya han surgido complicaciones. La patogenia de las complicaciones diabéticas no es bien conocida. Por término medio, las complicaciones de la DM se desarrollan entre 15 y 20 años después del debut de la enfermedad, aunque hay pacientes que tienen complicaciones ya en el momento del diagnóstico y otros que nunca las desarrollan[7]. Probablemente existe una predisposición genética y ésta sea multifactorial. Los pacientes diabéticos presentan un incremento del riesgo de sufrir enfermedad cardiovascular e infarto entre 2 y 4 veces más que los pacientes no diabéticos[33].

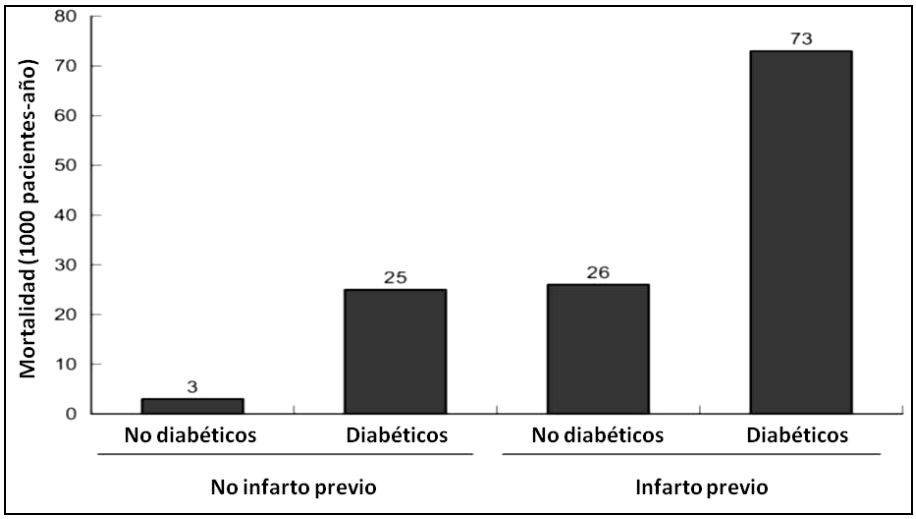

Figura 3. Tasa de mortalidad por causas cardiovasculares en sujetos diabéticos con o sin infarto agudo de miocardio previo[76].

Un estudio (Multiple Risk Factor Intervention Trial, MRFIT) en el que se evaluaban los diferentes factores de riesgo demostró un incremento en el riesgo de enfermedad cardiovascular en personas con DM tras ajustar por edad hipertensión, tabaco y colesterol[1]. En otros estudios se consideró el padecer DM como un equivalente de enfermedad coronaria[87]. Así, el riesgo de eventos cardiovasculares era tan alto en los diabéticos como en los pacientes con infarto previo[76] (Fig. 3). Las complicaciones asociadas a DM se pueden dividir en vasculares y no vasculares.

Complicaciones vasculares:

A. Microvasculares: en este caso la DM afecta a vasos de pequeño calibre. La complicación microvascular más frecuente en los pacientes diabéticos es la retinopatía diabética, principal causa de ceguera adquirida en el mundo. Además, se incluyen la nefropatía diabética como causa más común de enfermedad renal terminal, y la neuropatía diabética, que se manifiesta como disfunción del nervio periférico con compromiso sensitivo, motor y autonómico.
B. Macrovasculares: Las complicaciones macrovasculares se deben a una aterosclerosis acelerada de los vasos sanguíneos medianos y grandes. La principal estructura dañada por la hiperglicemia es el endotelio, debido a un desequilibrio entre la vasoconstricción y la vasodilatación mediada por óxido nítrico, que ocasiona la disfunción endotelial[115]. Estudios realizados en pacientes DM muestran que existe una correlación directa entre el grosor de la media e íntima carotídea y el grado de resistencia a insulina, hiperglicemia e hipertrigliceridemia post-prandial[115]. La enfermedad arterial periférica es quince veces más frecuente en pacientes con DM, afecta a ambos sexos y aumenta con la edad y duración de la DM. Otra complicación es la enfermedad cerebrovascular, también más frecuente en pacientes con DM2[3].

Complicaciones no vasculares:

Existen además complicaciones no vasculares asociadas a DM, como gastroparesia diabética, disfunción sexual y afecciones de la piel que no serán objetivo en esta tesis. Sin embargo, el efecto directo de la DM sobre el corazón puede condicionar el desarrollo y la evolución de la enfermedad.

2. Diabetes y miocardio

La DM puede afectar directamente al corazón actuando sobre su estructura y función en ausencia de hipertensión arterial y enfermedad coronaria, dando lugar a la denominada miocardiopatía diabética (MCD)[20]. El miocardio diabético no hipertenso se caracteriza por una hipertrofia y disfunción diastólica y/o sistólica en el ventrículo izquierdo (VI) debido a cambios

estructurales y funcionales[69,53]. Puede presentar una disminución de la fracción de eyección (FE) con un incremento del volumen y diámetro del VI. Las manifestaciones clínicas más representativas son disnea, arritmia, dolor torácico atípico y mareos[159]. Tras un infarto de miocardio el corazón diabético es además más susceptible a otras patologías. Los pacientes con DM muestran una hiperquinesia compensatoria menor en la zona no infartada que los no diabéticos[53] y una mayor incidencia de insuficiencia cardiaca[149]. La detección temprana de estas alteraciones puede jugar un papel importante en la evolución del paciente. Lamentablemente, en la actualidad no existe ningún tratamiento específico para la MCD.

2.1. ALTERACIONES EN LA FUNCIÓN CARDIACA

2.1.1 Disfunción diastólica

Mediante el uso de las técnicas de ecocardiografía-Doppler se ha observado una prevalencia de la disfunción diastólica en el 40-75% de individuos con DM1 y DM2 sin enfermedad coronaria[24]. Del mismo modo, se encontró disfunción diastólica en modelos animales de DM2, tales como ratones ob^-/ob^- (carentes del gen de leptina) y ratas ZDF (Zucker Diabetic Fatty; carentes del gen del receptor de leptina) tanto *in vivo* (por ecocardiografía)[4] como *ex vivo*[5].

Se han propuesto varios mecanismos para explicar la disfunción diastólica asociada a DM. El deterioro de la recaptación de calcio asociada a la disfunción contráctil podría ser uno de los implicados[166]. Del mismo modo, y coherente con el papel del calcio en la disfunción diastólica, la reducción de la contractilidad de los cardiomiocitos aislados de ratones db^-/db^- (carentes del gen del receptor de insulina) sedentarios se asoció con la liberación de Ca^{2+} del retículo sarcoplásmico y disminución de la presión sistólica y diastólica[5].

2.1.2 Disfunción sistólica

La disfunción sistólica es una manifestación más tardía de la afectación del miocardio en DM, y, en general, se desarrolla después de la disfunción diastólica. La disfunción sistólica puede no detectarse con el uso de la técnica estándar de ecocardiografía bidimensional. Sin embargo, utilizando el análisis Doppler tisular y las mediciones de la velocidad sistólica máxima, se han visto anomalías sutiles en la función sistólica en el 24% de los pacientes con DM después de excluir a los pacientes con enfermedad coronaria o hipertrofia del VI[52,195]. Además, mediante el estudio de curvas de presión-volumen también se ha podido detectar disfunción sistólica, manifestada como un retraso en la relajación del VI, en pacientes con DM1, o mayor rigidez del VI, en pacientes con DM2[141].

2.1.3 Deterioro de la reserva contráctil

La MCD puede estar presente incluso en sujetos asintomáticos con dimensiones y función normal del VI en reposo. Sin embargo, en algunos de estos individuos en la etapa inicial de la enfermedad, la disfunción del VI puede estar desencadenada por el ejercicio físico[158]. Estudios posteriores revelaron una alteración inducida por el ejercicio físico en el desarrollo de hipertensión sistólica en individuos con DM1 y DM2, sin evidencia de neuropatía autonómica o isquemia miocárdica, y con parámetros ecocardiográficos normales en reposo (incluyendo las mediciones de Doppler tisular) al inicio del estudio[75].

2.2. ALTERACIONES TISULARES EN EL MIOCARDIO DIABÉTICO

La hiperglicemia e hiperlipidemia inducidas en DM producen alteraciones metabólicas y cambios en la función de la membrana plasmática ya en los primeros días, cambios en la función contráctil en las primeras semanas, y cambios morfológicos y funcionales en el corazón durante los meses posteriores de la enfermedad (Fig. 6)[22]. Procesos como inflamación, hipetrofia, apoptosis y fibrosis han sido descritos en el miocardio DM1 y DM2. En los siguientes apartados profundizaremos en los mecanismos que subyacen estos procesos (Fig. 7).

2.2.1 Inflamación

La respuesta inflamatoria es un proceso relevante en la progresión del fallo cardiaco en MCD. En pacientes diabéticos se ha descrito la presencia de marcadores inflamatorios circulantes no específicos, como la proteína quimioatractante de monocitos-1 (MCP-1), IL-6, TNFα, troponina o proteína C-reactiva, asociados a disfunción cardiaca[144]. De hecho, en un estudio de Ray *et al.* observaron que los pacientes diabéticos presentaban un estado inflamatorio generalizado comparado con los pacientes no diabéticos, incluso en el contexto de síndrome coronario agudo[177]. Tschöpe *et al.* han mostrado en un modelo de DM1 experimental inducida por streptozotocina que en estadios tempranos del daño existe un incremento del número de células inmunocompetentes en el corazón. Esta inflamación se acompañaba de un incremento en la expresión ICAM-1 (intracellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) y otras moléculas de adhesión[185]. Tanto células infiltrantes como cardiomiocitos[193] son capaces de producir citoquinas proinflamatorias como IL-1, TNF-α[153]. Estas citoquinas pueden, a su vez, estimular la expresión de más móleculas de adhesión y, así, retroalimentar el sistema[99]. Las células cardiacas expresan receptores para las citoquinas pro-inflamatorias IL-1β y TNF-α que podrían inducir la activación de NF-κB[23]. NF-κB es un factor de transcripción capaz de aumentar la expresión de genes proinflamatorios entre otros[185]. Aunque la inflamación en humanos no está completamente demostrada[155], los

estudios en animales indican que la presencia de leucocitos en el corazón diabético y la activación de NF-κB puede jugar un papel crucial. En este sentido, muchos de los efectos beneficiosos de los tratamientos para MCD incluyen la mejora del estado inflamatorio y disminución de NF-κB[16] (Fig. 4). En este sentido, existe un equilibrio entre la formación de moléculas oxidantes y los sistemas antioxidantes de la célula. Además, el estado redox será también determinante para la producción de citoquinas proinflamatorias. Varios estudios sugieren que la angiotensina II (AngII) está implicada en el inicio y progresión del proceso inflamatorio en la MCD[184]. AngII es capaz de activar células inflamatorias circulantes que posteriormente se adhieren al endotelio y migran al interior de las distintas células. La AngII posee propiedades quimioatractantes e induce la sobreexpresión de citoquinas proinflamatorias. *In vivo*, AngII incrementa la producción de TNF-α e IL-6, y activa al factor nuclear NF-κB; efectos asociados con la presencia intersticial de células inflamatorias en el corazón[190].

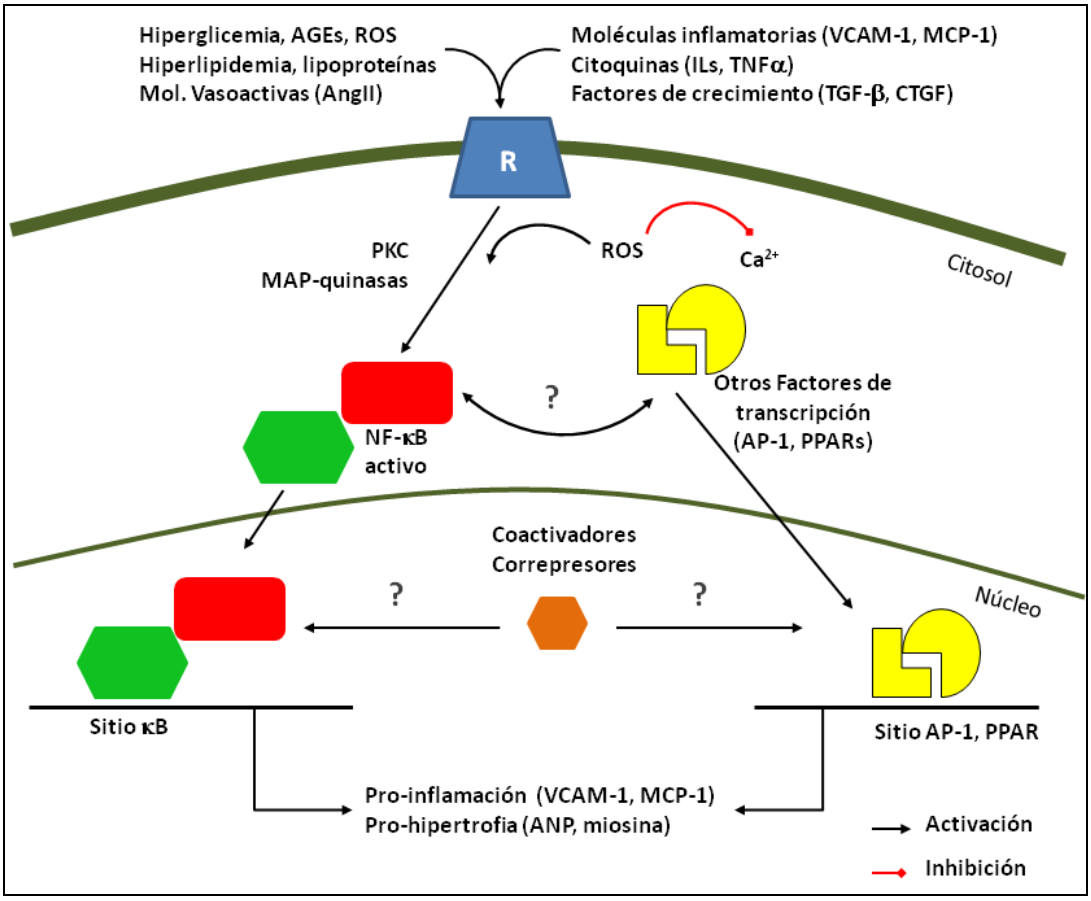

Figura 4. Expresión génica en inflamación e hipertrofia del corazón diabético. En DM, la hiperglicemia, hiperlipidemia y otros factores inducen citoquinas y moléculas inflamatorias que activan NF-kB y otros factores de transcripción. Estos factores regulan la expression de factores relacionados con la inflamación e hipertrofia. R: receptor.

2.2.2 Hipertrofia

La hipertrofia cardiaca constituye una de las principales respuestas del cardiomiocito a estímulos mecánicos y neurohormonales que permite al miocito generar mayor trabajo y mejorar la contractilidad cardiaca. Esta acción compensatoria, sin embargo, se ve en algún momento sobrepasada por el estrés biomecánico, lo que puede dar lugar a la aparición de insuficiencia cardiaca[35]. El remodelado cardiaco hace referencia a los cambios en la geometría del VI y en la masa del corazón (disminuye el número de cardiomiocitos, aunque incrementa el volumen), que comprometen de manera desfavorable la función del músculo cardíaco[42].

El patrón de hipertrofia varía según el contexto etiológico en el que se produce[154]. Así, por ejemplo en la sobrecarga de presión, las proteínas del sarcómero que se están sintetizando en exceso se agrupan en paralelo, por lo que los cardiomiocitos crecen en grosor y el ventrículo crece de manera concéntrica y aumenta el cociente grosor de la pared/diámetro de la cámara ventricular[106]. Se han identificado algunos de los factores que regulan el proceso de agrupación de las proteínas sarcoméricas. Las vías intracelulares que se activan en estos procesos dan lugar a una re-expresión génica del programa fetal activando genes tempranos (c-jun, c-fos, c-myc) y genes fetales [péptido natriurético atrial (ANP), cadena pesada de la miosina β (β-MHC) y alfa-actina esquelética (SKA), entre otros], que también pueden ser utilizados como marcadores de la respuesta hipertrófica[31]. Además, diversos factores humorales pueden actuar como estímulo hipertrófico en el cardiomiocito. En este sentido, un primer grupo está compuesto por los factores de crecimiento TGF-β (factor de crecimiento transformante-β), FGF (factores de crecimiento de fibroblastos) e IGF (factor de crecimiento similar a la insulina)[130,107]. Un segundo tipo de estímulo para el crecimiento del cardiomiocito procede de la estimulación de receptores AT1 para AngII (Fig. 4) y ET para endotelina-1[83]. Además, evidencias recientes han relacionado la desactivación de la vía de señalización de PPARα (*peroxisome proliferator-activated receptor-*α) con la menor expresión génica de enzimas implicadas en la oxidación de ácidos grasos en el corazón hipertrófico. En ratas tratadas con fenobibrato, agonista de PPARα, se bloqueó la hipertrofia secundaria a una sobrecarga de presión[89]. Algunos estudios con microarrays han revelado además un cambio coordinado en la expresión de genes implicados en la transducción de señal, función metabólica, estructura y motilidad en DM[170]. Estos cambios podrían estar mediados por elementos del citoesqueleto como la desmina o la actina[36].

2.2.3 Fibrosis

En condiciones fisiológicas los niveles de síntesis y degradación de matriz extracelular (MEC), principalmente colágenos, están en equilibrio. Sin embargo, durante el desarrollo de la

MCD, las alteraciones hemodinámicas o bioquímicas locales pueden modificar este balance e incrementar la produción de MEC y/o disminuir su degradación implicando a los cardiomiocitos y fibroblastos[48] como células productoras. La fibrosis cardiaca se clasifica en dos tipos: fibrosis reparadora y reactiva. La fibrosis reparadora ocurre tras un infarto de miocardio. La pérdida de cardiomiocitos por apoptosis o necrosis precede a la hipertrofia de las miocitos supervivientes, la hiperplasia de fibroblastos y deposición de material fibrótico. Sin embargo, la fibrosis reactiva generalmente ocurre en ausencia de pérdida célular como resultado de cambios mecánicos o bioquímicos locales, como por ejemplo, la hipertensión arterial. Mientras que la fibrosis reparadora suele afectar casi exclusivamente a la MEC intersticial, la fibrosis reactiva puede ser observada en el compartimento intersticial así como en el espacio perivascular. En este sentido, en la MCD humana aparece tanto fibrosis intersticial como perivascular[145]. La progresión de la fibrosis en la MCD es debida al aumento de la síntesis de MEC (colágenos y fibronectina) y una menor degradación por parte de las metaloproteasas (MMP-2)[14] (Fig. 5). Los factores de crecimiento ganan protagonismo en la fibrosis reactiva. La hiperglicemia, además, es capaz de estimular la expresión de TGF-β en fibroblastos cardiacos[84]. TGF-β es el primer mediador de la síntesis de MEC y de la disminución de su degradación. Mediante la unión a su receptor, TGF-β fosforila proteínas Smads e induce la activación de diferentes factores de transcripción (ej: AP-1) para aumentar la expresión de MEC. Otro factor profibrótico importante en esta regulación es el factor de crecimiento de tejido conectivo [28,28], el cual tiene propiedades multifuncionales que incluyen la regulación y síntesis de MEC[192]. CTGF también activa la expresión de las proteínas TIMPs, inhibidores de metaloproteinasas (MMPs), cuya función es la degradación de la matriz extracelular. Además, CTGF puede ser modulado por la bioactividad de otras citoquinas, en especial TGF-β, sugiriendo que TGF-β es necesario para que CTGF produzca su efecto [123].

AngII desempeña un papel importante en la fibrosis cardiaca asociada a MCD (Fig. 5), regulando el crecimiento celular y la síntesis de MEC. Muchos estudios han demostrado que AngII participa en la fibrosis cardiaca a través de la producción endógena de los factores CTGF y TGF-β. En el corazón existe una interesante relación entre AngII y TGF-β. AngII estimula la expresión de TGF-β, y además el bloqueo de TGF-β modifica algunas respuestas de AngII, entre ellas la regulación de la MEC. TGF-β ejerce sus acciones pro-fibróticas a través de la unión a sus receptores que fosforilan Smad 2 y Smad 3, y éstos reclutan a Smad4. Este complejo ya fosforilado se une a las secuencias promotoras de los genes diana (Fig. 5)[59]. Por otro lado, la AngII es capaz de activar Smad a través del receptor AT1 y MAPK y regular la expresión génica a través de AP-1[78,181].

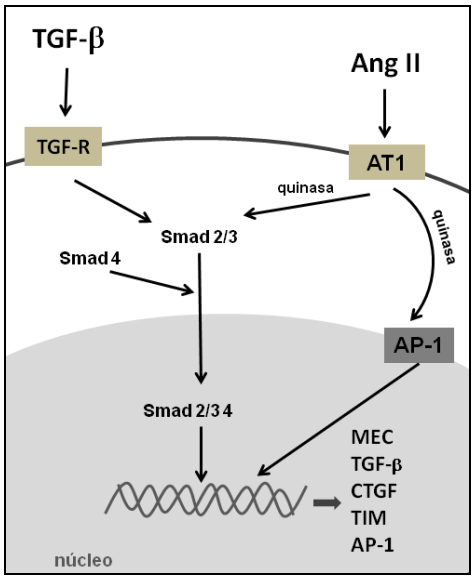

Figura 5. Mecanismos profibróticos activados en la MCD. (MEC: Matriz extracelular)

2.2.4 Apoptosis

La apoptosis constituye una forma de muerte celular programada con características morfológicas y dinámicas distintas a la muerte celular por necrosis[138]. Las alteraciones de la regulación de la apoptosis pueden participar en el desarrollo de numerosas patologías[66]. En estudios de diabetes en humanos y animales se han detectado altos niveles de apoptosis en miocitos, células endoteliales y fibroblastos[29]. La apoptosis puede ocurrir por varias vías en el microambiente diabético. Principalmente la apoptosis en la MCD puede ser vía extrínseca o intrínseca. La vía extrínseca se inicia por estímulos que actúan desde el exterior de la célula. Éstos incluyen citoquinas como TNFα y ligando de Fas (FasL) que actúan a través de sus receptores, TNFR1 y Fas, asociados a mediadores con secuencias de dominios de muerte, TRADD y FADD (*TNFR1-associated death domain, Fas-associated protein with death domain*). Estos mediadores activan procaspasas específicas como caspasa 8, que a su vez induce proteolisis de caspasas efectoras como la caspasa 3. La caspasa 3 entrará en el núcleo y degradará la estructura cromosómica[12] (Fig. 6). La activación de caspasa 8 puede además activar Bid que se transloca a la mitocondria para amplificar la respuesta apoptótica mediada por la vía intrínseca.

La vía intrínseca o mitocondrial se induce a través de estímulos celulares endógenos, como proteínas de la familia del gen del linfoma de células B (Bcl-2). Algunas de estas proteínas (como la proteína X asociada a Bcl-2, Bax) alteran la integridad estructural y funcional de la mitocondria, facilitando la liberación del citocromo C al citosol, donde estimulará al factor activador de la proteasa apoptótica 1 (APAF-1). APAF-1 formará el apoptosima, junto a caspasa 9, y este activará a caspasa 3. Por el contrario, otras proteínas de la familia (como Bcl-2) preservan la integridad mitocondrial. Así pues, del balance entre proteínas

proapoptóticas y antiapoptóticas de la familia Bcl-2 resultará la inducción de este mecanismo de muerte celular[41]. Hay un tercer mecanismo de activación de apoptosis que es a través del retículo endoplasmático.

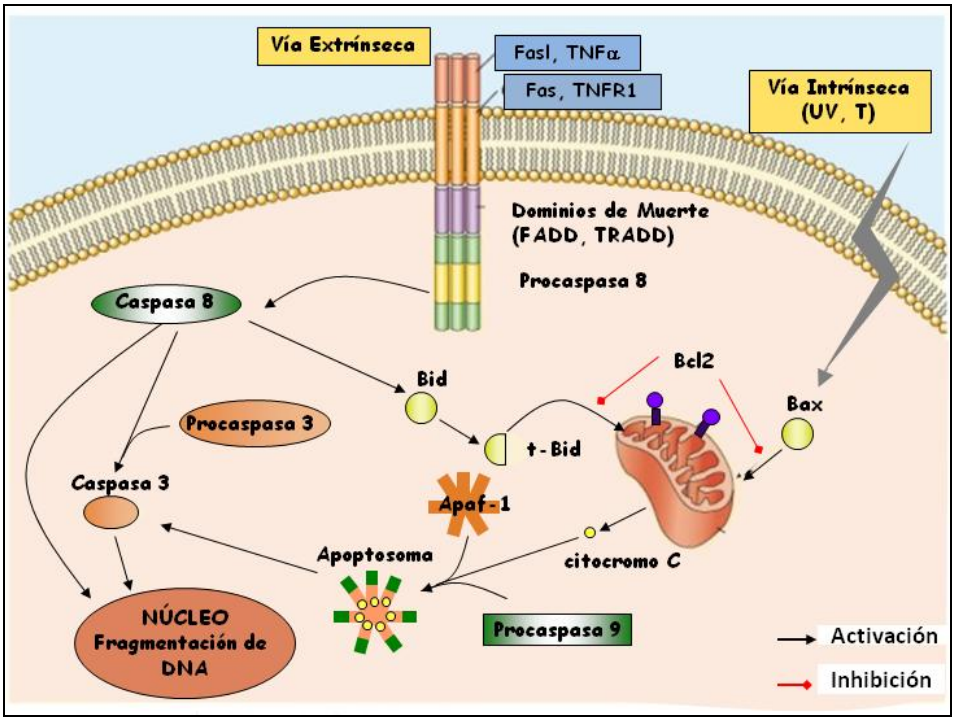

Figura 6. Mecanismos de apoptosis en la MCD. A la izquierda vía extrínseca o de receptores letales que conlleva el procesamiento de la caspasa iniciadora, caspasa 8, que a su vez induce proteolisis de caspasas efectoras como la caspasa 3, generando el proceso de apoptosis. A la derecha vía intrínseca o mitocondrial iniciada por factores proapoptóticos o de estrés que convergen en la mitocondria, dando lugar a la activación de proteínas proapoptóticas de la familia Bcl-2 como Bax. (TNFR1: receptor de TNFα 1).

La apoptosis en el cardiomiocito diabético puede ser consecuencia de un metabolismo celular anormal y de defectos en la mitocondria, el sarcolema o el retículo endoplasmático. La hiperglicemia e hiperlipidemia participan en el proceso induciendo la expresión de citoquinas letales, la formación de ROS y productos secundarios, la acumulación de ácidos grasos libres, el estrés en el retículo endoplasmático y la desestabilización de la membrana mitocondrial[176].

2.3. ATERACIONES MOLECULARES DEL MIOCARDIO DIABÉTICO

2.3.1 Glicotoxicidad

La inicial y principal alteración en la MCD es el exceso de glucosa circulante. Este exceso se produce por una carencia en la respuesta a insulina. La hiperglicemia produce alteración directa en el músculo cardiaco[165]. En condiciones fisiológicas, la entrada de glucosa en el corazón viene determinada por los niveles adecuados de insulina que favorecen la translocación del transportador de glucosa 4 (GLUT-4) a la superficie celular. La glucosa es utilizada como fuente de energía mediante su degradación por glicolisis, seguida del ciclo de los ácidos tricarboxílicos (TCA) y la cadena transportadora de electrones (Fig. 8). Por otro lado, la insulina, además de promover la entrada de glucosa al interior de la célula, también es capaz de bloquear la liberación de ácidos grasos desde el tejido adiposo disminuyendo, así, sus niveles plasmáticos y desbloqueando la inhibición de la glicolisis por metabolitos de ácidos grasos (Fig. 8)[142]. La glucosa suprimiría la oxidación de ácidos grasos de cadena larga mediante la inhibición de CPT-1 mediada por malonil-CoA[150]. Sin embargo en el corazón diabético, el exceso de glicolisis también da lugar a la formación de ROS mediante la respiración mitocondrial. ROS atenúa la ATPasa dependiente de calcio del retículo sarcoplásmico (SERCA) derivando en disfunción contráctil y MCD[171].

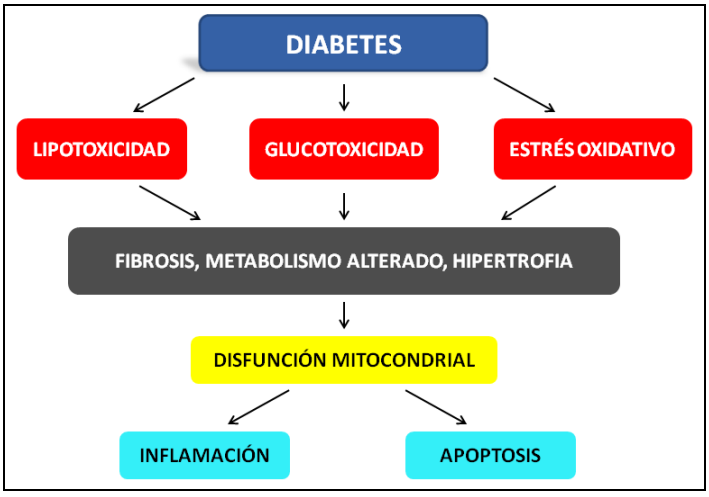

Figura 7. Alteraciones metabólicas y funcionales del corazón diabético.

2.3.2 Lipotoxicidad

En pacientes DM se ha observado un incremento de lípidos circulantes que se tranduce en una acumulación de éstos en los tejidos periféricos como el corazón[182]. Los recientes avances en espectroscopía de resonancia magnética nuclear han permitido la evaluación no invasiva de contenido de triglicéridos y otros ácidos grasos en el miocardio[116]. En el

cardiomiocito normal, los ácidos grasos cumplen muchas funciones esenciales. Estas funciones incluyen aportación de energía, transducción de señales (como la activación de proteín-quinasa C), activación de factores de transcripición nucleares [43] y formación de membranas biológicas[143,50]. Sin embargo el exceso de lípidos circulantes produce un incremento de su ingesta (gracias a receptores FAT o FATP) y una excesiva acumulación en el citosol, que se asocia con resistencia a insulina y cardiotoxicidad en DM (Fig. 8). No es de extrañar, por tanto, que numerosos autores hayan sugerido que la DM es más una alteración en el metabolismo lipídico que del metabolismo de la glucosa.

Los PPARs son miembros de una superfamilia de receptores nucleares que actúan como factores de transcripción. Tras la unión con sus ligandos, los PPARs forman heterodímeros con otros receptores RXRs (*9-cis retinioc acid-activated receptors*) para interaccionar con secuencias promotoras del ADN de genes diana. Los ácidos grasos de cadena larga y sus derivados como eicosanoides sirven como ligandos endógenos para PPAR[110]. Existen tres miembros en la familia PPAR (α, β/δ y γ) con diferentes y/o solapantes funciones. PPARα aparece principalmente en tejidos con alta capacidad para la oxidación de ácidos grasos tales como el corazón, tejido adiposo pardo, músculo esquelético e hígado[54]. PPARα incrementa la expresión de genes implicados en la ingesta, transporte y degradación de ácidos grasos. PPARα también está implicado en el control de genes proinflamatorios al interaccionar con NF-κB[54]. PPAR β/δ es expresado ubicuamente, pero con altos niveles en corazón, músculo esquelético y cerebro, participando también en la sobrerregulación de genes de la oxidación de ácidos grasos[26]. Finalmente, PPARγ se expresa principalemente en el tejido adiposo[19]. Tiene un papel fundamental en la diferenciación de los adipocitos y en el almacenamiento de grasa. Además, existen cofactores que se unen al heterodímero PPAR/RXR tales como CBP/p300, PBP/TRAP220, PGC-1 y SRC-1. De éstos, PCG-1 es altamente expresado en el corazón y vinculado además con la biosíntesis mitocondrial[44].

Por otro lado, los ácidos grasos también son capaces de alterar el metabolismo, función y expresión génica celular mediante un mecanismo independiente de PPARα. Una vez en el interior de la célula, los ácidos grasos son activados mediante un enlace tioéster con el coenzima A (CoA). Este complejo es transportado hacia el interior de la mitocondria via carnitina palmitoiltransferasa 1 (CPT-1), para ser degradado mediante la β-oxidación. Cuando el transporte de ácidos grasos en el miocito excede la capacidad oxidativa de la mitocondria, los niveles citosólicos de acil-CoA son incrementados y pueden desviarse hacia la formación de diacilglicerol (DAG)[105] y ceramidas. DAG es un activador alostérico de isoformas de proteín-quinasa C (PKC) que inactivan la señal de insulina. Por otro lado, las ceramidas pueden provocar apoptosis a través de la activación de Bax y formación de poros mitocondriales.

Finalmente, la β-oxidación porduce también ROS y, así, desacople y disfunción mitocondrial[194] (Fig. 8).

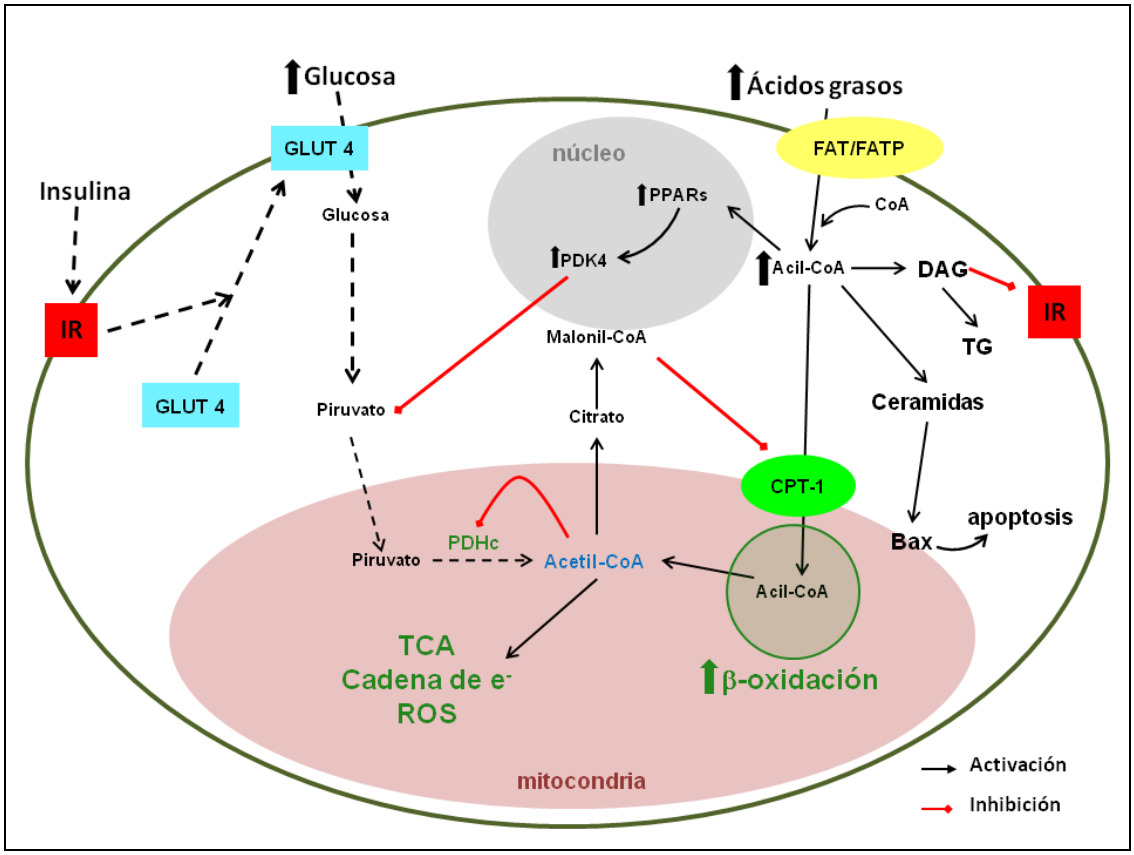

Figura 8. Regulación del metabolismo lipído y glucídico en el corazón diabético (TCA: ciclo de los ácidos tricarboxílicos, CPT-1: carnitina palmitoiltransferasa 1, DAG: diacilglicerol, FAT/CD36: traslocasa de ácidos grasos, GLUT4: transportador de glucosa 4, IR: receptor de insulina, PDH: piruvato deshidrogenasa, PDK4: piruvato deshidrogenasa quinasa 4, ROS: especies reactivas de oxígeno). Las flechas punteadas indican disminución. En el cardiomiocito diabético la entrada y oxidación de la glucosa está bloqueada, por lo que los ácidos grasos son utilizados como única fuente de energía.

2.3.3 Estrés oxidativo y disfunción mitocondrial

El exceso de glucosa en plasma produce glicoxilación de proteínas (ej. colágeno) y su reticulación de tal manera que se forman AGEs. Los AGEs se unen a RAGES (receptro de AGE) y activan NOX para producir ROS. Además los RAGEs activan PKC, el cual fosforila proteínas encargadas de la contracción cardiaca. La glucosa activa directamente iNos[34] y, así, ROS. Estudios en humanos y animales DM sugieren una estrecha relación entre el estrés oxidativo y la sobrecarga de lípidos[146]. El estrés oxidativo está aumentado en corazones aislados de ratones diabéticos, junto con la acumulación de lípidos y la β-oxidación mitocondrial[21] (Fig. 8). ROS en exceso si no es neutralizado por los sistemas antioxidantes de

la célula produce despolarización de las membranas mitocondriales y formación de poros. La matriz mitocondrial se hincha por entrada de excesiva agua y salida de solutos. También se produce la salida de citocromo c, formación del apoptosoma y apoptosis. ROS también activa Bax y puede alterar la expresión génica al oxidar el ADN mitocondrial.

El incremento de ROS no puede ser explicado únicamente a través del aumento de ácidos grasos. Aunque un alto porcentaje de ROS celular total es generado por la mitocondria, hay sistemas enzimáticos capaces de generar ROS en el citosol, como la NADPH oxidasa, que puede ser modulada en DM[102,161]. En ratas ZDF, el incremento de ROS citosólico también contribuyó a la activación de NF-κB e inflamación[11]. Además, mientras que existen evidencias del incremento de la producción de ROS en DM, el papel de las defensas antioxidantes es controvertido. Los niveles de actividad/expresión de glutatión peroxidasa, superoxido dismutasas (SODs) o catalasas se han encontrado tanto aumentados[187,188] como disminuidos[114,8]. Desafortunadamente la capacidad antioxidativa de estas defensas no es capaz de neutralizar la producción de ROS en el corazón DM[162,191].

3. Implicación del sistema renina-angiotensina-aldosterona en la MCD

Tras el descubrimiento de los principales componentes del sistema Renina-Angiotensina-Aldosterona (SRAA), cuya existencia fue postulada hace más de 100 años, se inició una etapa de estudio de su rol patogénico en enfermedades cardiovasculares. La renina se libera desde las células yuxtaglomerulares en forma inactiva en respuesta a la disminución de volemia o presión arterial (Fig. 9). En la circulación general, actúa sobre el angiotensinógeno dando lugar a la angiotensina I. Ésta, mediante la enzima convertidora de angiotensina (ECA), se transforma en AngII, como péptido multifuncional con función vasoconstrictora. AngII aumenta la resistencia vascular periférica, disminuye la eliminación renal de agua y sal y estimula la liberación de aldosterona. La aldosterona se sintetiza en la corteza de la glándula suprarrenal y es un mineralocorticoide que induce la reabsorción de sodio y agua en los túbulos distal y colectores de las nefronas. Existe un sistema local del SRAA además del circulante de gran importancia en algunos órganos como el corazón[20].El SRAA es un sistema cardiovascular de respuesta al estrés, que además de regular la actividad renal vasomotora y mantener la homeostasis, puede tener consecuencias fisiopatológicas derivadas de una estimulación elevada como en la generación de hipertensión arterial. Por otro lado, la AngII, además de modular la contracción celular, regula el crecimiento, apoptosis y diferenciación celular. Influye en la migración y la deposición de la MEC, es proinflamatoria, estimula la producción de factores de crecimiento como CTGF y TGF-β[122,28] y transactiva receptores de factores de

crecimiento[93], promoviendo la proliferación celular. La AngII estimula citoquinas como IL-6 y TNF-α y metaloproteinasas[47]. La aldosterona, además estimula la fibrosis e hipertrofia[183], predispone al estrés oxidativo inflamación[147]. De hecho la aldosterona estimula la producción de AngII. La aldosterona promueve las acciones de la AngII y fibrosis en el miocardio diabético, mediante activación de factores pro-fibróticos[133]. La aldosterona se une a receptores mineralocorticoides citoplasmáticos encontrados en células cardiacas, entre otras[63,37]. Los efectos de la aldosterona en cuanto a la fibrosis, hipertrofia e infamación son similares a los hallados en AngII. Por ello, cada vez hay más evidencias científicas que sugieren que el bloqueo de los mineralocorticoides podría suponer beneficios terapéuticos que irían más allá de su efecto hipotensor[13]. Además, estudios recientes han revelado que el bloqueo de la aldosterona podría tener un efecto beneficioso independiente de la AngII[137,197].

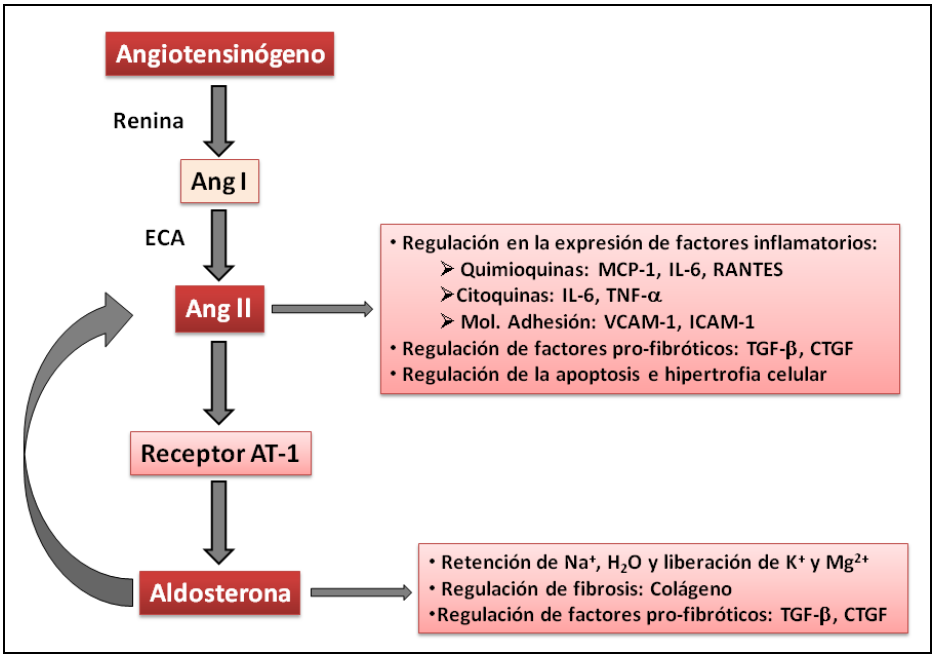

Figura 9. Sistema renina-angiotensina-aldosterona.

3.1. ANTAGONISTAS DE LOS RECEPTORES DE ALDOSTERONA: EPLERENONA

En 1999, en el estudio RALES[132], se observó que la espironolactona, agregada al tratamiento estándar con inhibidores de la enzima convertidora de angiotensina (IECAs), beta bloqueantes, digoxina y diuréticos, disminuía la mortalidad total en pacientes con insuficiencia cardiaca congestiva de grado severo y disfunción sistólica del VI[82]. Sin embargo, la espironolactona es un antagonista no selectivo de la aldosterona capaz de unirse a otros receptores esteroideos[118,167] dando lugar a efectos no deseados (ginecomastia, hipertrofia prostática y disfunción eréctil). En 2001 se desarrolló la eplerenona, el primer antagonista

selectivo de la aldosterona[40]. Este compuesto causaba menos efectos adversos comparado con la espironolactona presentando la misma potencia para bloquear los receptores mineralocorticoides[167]. Aldosterona, cortisol y corticosterona pueden unir con similar afinidad a los MR. Sin embargo la aldosterona puede poseer importantes propiedades reguladoras en el sistema cardiovascular y, así, en la MCD. El estudio EPHESUS (*Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study*)[134] demostró en pacientes con disfunción sistólica post-infarto, que el bloqueo temprano de la aldosterona con eplerenona disminuía un 15% la mortalidad global, un 17% la mortalidad/hospitalización cardiovascular y un 21% la muerte súbita por causa cardiaca[82] en comparación con placebo.

Interesantemente estos resultados no fueron atribuibles solo a la acción de la eplerenona sobre el riñón. En este sentido, la eplerenona podría mejorar estos eventos actuando directamente sobre las células cardiacas.

3.2. EPLERENONA Y CORAZÓN DIABÉTICO

Los estudios de los posibles efectos favorables de la eplerenona sobre la DM y el daño que ésta produce sobre el corazón son muy recientes. Muchos de ellos son análisis *post hoc* sobre la población diabética del estudio EPHESUS como el de O'Keefe *et al.* que describe que la eplerenona produce una mayor la supervivencia en los pacientes diabéticos[129]. La reducción del riesgo relativo de muerte era similar a la observada en el cohorte de pacientes no diabéticos del estudio EPHESUS, aunque la disminución del riesgo absoluto con eplerenona es superior (debido a las mayores tasas de eventos cardiovasculares en pacientes diabéticos)[123]. A nivel experimental, en roedores diabéticos se ha observado que la activación de los receptores mineralocorticoides podría inducir el desarrollo de inflamación cardiaca[64]. Consecuentemente, se produce una mejora en la función cardiaca[85]. Guo *et al* mostraron en ratones diabéticos y obesos que la eplerenona reducía los marcadores de inflamación como TNF-α, MCP-1 e IL-6 en el corazón[172,74]. Por otro lado, el antagonismo del receptor de la aldosterona reducía la hipertrofia ventricular izquierda[134]. Finalmente, el tratamiento con eplerenona aminoró la fibrosis inducida por DM en un modelo con ratones *db/db* (carentes del receptor de leptina) disminuyendo el depósito de matriz extracelular y la expresión de TGF-β[73].

HIPÓTESIS

III. HIPÓTESIS

- Las *diabetes mellitus* experimentales tipo 1 y tipo 2 podría inducir alteraciones en el miocardio incluyendo procesos de inflamación, hipertrofia, apoptosis y fibrosis cardiaca. La coexistencia de hipertensión enfatizaría estos eventos.

- Mediante un abordaje proteómico, podríamos identificar nuevos mediadores y/o rutas intracelulares implicadas en el desarrollo de la miocardiopatía diabética.

- El bloqueo de los receptores de mineralocorticoides podría atenuar la respuesta apoptótica del miocardio DM2/obeso y mejorar la función cardiaca.

OBJETIVOS

IV. OBJETIVOS

El objetivo principal de esta tesis fue la caracterización de las alteraciones principales que acontecen en el corazón diabético de rata, estudiando la implicación de mediadores de procesos asociados y analizando el potencial efecto terapéutico del bloqueo de los receptores de mineralocorticoides.

Los objetivos concretos fueron:

1) Desarrollo de un modelo de DM1 inducido por streptozotocina en ratas normotensas e hipertensas:
    i) Caracterizando y estudiando las respuestas tisulares y celulares de sus corazones, y cardiomiocitos en cultivo.
    ii) Analizando el patrón de expresión proteico de cada corazón.
    iii) Prediciendo potenciales vías moleculares implicadas.

2) Desarrollo de un modelo genético de DM2/obesidad en rata:
    i) Caracterizando y estudiando las respuestas tisulares y celulares de estos corazones y cardiomiocitos en cultivo, en particular el proceso de apoptosis y el potencial efecto atenuador de la eplerenona.

MÉTODOS

Y RESULTADOS

V. MÉTODOS Y RESULTADOS

1. FIBROSIS Y APOPTOSIS CARDIACA, PERO NO INFLAMACIÓN, ESTÁN PRESENTES EN LA DIABETES EXPERIMENTAL CRÓNICA.

El daño cardiaco en DM puede ser directamente inducido por DM y enfatizado por la coexistencia de enfermedad coronaria y/o hipertensión[20]. Hipertrofia, fibrosis, apoptosis e inflamación han sido descritas en estadios tempranos del daño miocárdico en DM1 experimental (hasta 10 semanas)[139,57,185]. En nuestro modelo de DM1 crónica (22 semanas) se observó un aumento en la expresión de factores profibróticos, TGF-β, CTGF y proteínas de matriz extracelular, así como activación de factores de transcripción ligados a TGF-β como p-Smad 4 y AP-1. Además, apoptosis y sobreexpresión de moléculas pro-apoptóticas FasL, Fas, Bax, y caspasa-3 mientras que Bcl2, proteína anti-apoptótica, estaba disminuida. Sin embargo, en DM1 crónica, el proceso inflamatorio estaba atenuado, posiblemente debido a la expresión local de moléculas anti-inflamatorias y anti-oxidantes como IL-10, catalasa y hemo-oxigenasa 1 (HO-1). En este sentido, en cardiomiocitos en cultivo, IL-10 y TGF-β bloquearon la expresión de genes pro-inflamatorios inducidos por exceso de glucosa. Por otro lado, en ratas hipertensas (SHR) observamos un aumento similar de factores pro-fibróticos y apoptóticos y además inflación y moléculas proinflamatorias. Interesantemente, en el miocardio de animales con coexistencia de DM1 e hipertensión se observó una activación de NF-κB, incremento del infiltrado inflamatorio y sobreexpresión de mediadores inflamatorios IL-1, TNF-α, MCP-1, VCAM-1, angiotensinógeno, y oxidantes (NOS-1), que estaban ausentes en DM1. Sin embargo, en un modelo agudo de DM1 e hipertensión, tanto las ratas DM1 como las hipertensas presentaron inflamación, además de leve fibrosis y apoptosis. Por tanto, fibrosis y apoptosis son fenómenos característicos de la lesión miocárdica en DM1 crónica experimental. Sin embargo, la inflamación solo está presente en hipertensión y DM1 temprana. La reducción local de los factores pro-inflamatorios y la expresión de anti-inflamatorios y moléculas antioxidantes podrían ser los responsables de este efecto.

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Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes

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Ares-Carrasco S, Picatoste B, Benito-Martín A, Zubiri I, Sanz AB, Sánchez-Niño MD, Ortiz A, Egido J, Tuñón J, Lorenzo O. Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes. *Am J Physiol Heart Circ Physiol* 297: H2109–H2119, 2009. First published October 9, 2009; doi:10.1152/ajpheart.00157.2009.—The aim of this paper is to study the myocardial damage secondary to long-term streptozotocin-induced type 1 diabetes mellitus (DM1). Normotensive and spontaneously hypertensive rats (SHR) received either streptozotocin injections or vehicle. After 22 or 6 wk, DM1, SHR, DM1/SHR, and control rats were killed, and the left ventricles studied by histology, quantitative PCR, Western blot, ELISA, and electromobility shift assay. Cardiomyocyte cultures were also performed. The expression of profibrotic factors, transforming growth factor-β (TGF-β_1), connective tissue growth factor, and matrix proteins was increased, and the TGF-β_1-linked transcription factors phospho-Smad3/4 and activator protein-1 were activated in the DM1 myocardium. Proapoptotic molecules FasL, Fas, Bax, and cleaved caspase-3 were also augmented. Myocardial injury in long-term hypertension shared these features. In addition, hypertension was associated with activation of NF-κB, increased inflammatory cell infiltrate, and expression of the mediators [interleukin-1β (IL-1β), tumor necrosis factor-α, monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, angiotensinogen, and oxidants], which were absent in long-term DM1. At this stage, the combination of DM1 and hypertension resulted in nonsignificant additive effects. Moreover, the coexistence of DM1 blunted the inflammatory response to hypertension. Anti-inflammatory IL-10 and antioxidants were induced in long-term DM1 and DM1/SHR hearts. Myocardial inflammation was, however, observed in the short-term model. In cultured cardiomyocytes, IL-10, TGF-β_1, and catalase blocked the glucose-stimulated expression of proinflammatory genes. Fibrosis and apoptosis are features of long-term myocardial damage in experimental DM1. Associated hypertension does not induce additional changes. Myocardial inflammation is present in hypertension and short-term DM1, but is not a key feature in long-term DM1. Local reduction of proinflammatory factors and expression of anti-inflammatory and antioxidant molecules may underlie this effect.

hypertension; inflammation; heart

DIABETES MELLITUS (DM) IS becoming a pandemic. In 2003, 194 million people had diabetes, and this number is predicted to increase by 72%, affecting 366 million in 2030. Of them, at least 10% have type 1 diabetes (DM1). More than 65% of diabetic patients die because of cardiovascular complications (29, 39). Cardiac damage in DM can be directly induced by DM and enhanced by the coexistence of coronary artery disease and hypertension (2, 14, 21).

Most information on pathogenic mechanisms in diabetic cardiomyopathy is derived from animal models (2, 28). Hypertrophy, fibrosis, apoptosis, and inflammation have been described in the early stages (up to 10 wk) of experimental DM1 myocardial injury (8, 9, 15, 28, 37, 38). The excess of glucose alters metabolic, structural, and contractile proteins and activates cellular responses. Upregulation of the local renin-angiotensin-aldosterone (RAA) system in DM has been shown to be associated with oxidative damage, cardiac cell apoptosis, and interstitial fibrosis (1, 6, 8, 9). Increased reactive oxygen species (ROS) generation and impaired antioxidant defenses could both contribute to oxidative stress in DM hearts (9, 14, 15). Transforming growth factor-β (TGF-β) is another factor overexpressed in DM1 myocardium and involved in fibrotic processes (3, 6, 14, 37). Caspase-3 activation, downregulation of antiapoptotic molecules, and inflammatory changes, such as leukocyte infiltration and adhesion molecules production, have also been described in the early experimental DM1 myocardium (13, 15, 38). Many of these features are shared by hypertensive cardiomyopathy (9, 19, 27). Some of them, such as the presence of fibrosis, have been confirmed in human myocardial biopsies (21, 35), but there is much less information on features such as local inflammation. The molecular mechanisms involved in these key processes are not fully elucidated, and there is not enough information on the long-term injury of the DM1 heart and its relationship with coexistent hypertension.

In this work, we hypothesized that long-term myocardial injury from persistent hyperglycemia may differ from short-term damage. In this sense, we have studied the fibrotic, apoptotic, and inflammatory events in long- and short-term streptozotocin (STZ)-induced DM1, hypertensive, and DM1/hypertensive myocardium. In long-term DM1, many features of short-term myocardial injury are maintained. However, the inflammatory process appears to be blunted, possibly due to the local expression of anti-inflammatory and antioxidant molecules. Moreover, at this stage, the effects of both combined DM1 and hypertension are, for the most part, not additive.

METHODS

Type 1 diabetic models in rats. Normotensive Wistar-Kyoto and spontaneously hypertensive (SHR) male rats (6 wk of age) received either two STZ injections (50 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle. As a result, there were four different groups ($n = 6–10$ per group): DM1 normotensive, SHR, DM1/SHR, and control (normotensive) rats. Weight, glycemia, systolic blood pressure (by tail-cuff method), and albuminuria were periodically measured. Final levels are shown in Supplemental Fig. 1. (The online version of this article contains supplemental data.) Insulin (1–4 IU sc, Insulatard NPH) was administrated

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weekly to prevent death, but keep blood glucose >400 mg/dl. After 22 wk of treatment (long-term DM1), rats were killed, and the left ventricles isolated. One-half of the sample was included in 4% paraformaldehyde for histological studies, and the other one-half was frozen in liquid N_2 for protein and RNA assays. Serum creatinine, measured at death, remained within normal limits in all groups (0.5 ± 0.08 mg/dl). Some 6-wk-old rats were similarly treated with STZ and followed for 6 wk (short-term DM1), and their left ventricles analyzed. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the Ethical Committee of the Hospital.

Cardiomyocyte culture. H9c2(2-1) is a permanent cardiomyocytes cell line derived from embryonic BD1X rat heart tissue (ATCC). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (BioWhittaker, Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (BioWhittaker), 100 IE/ml sodium penicillin (Yamanouchi Europe, Leiderdorp, The Netherlands), 100 μg/ml streptomycin (Radiopharma-Fisiopharma), 2 mM L-glutamine (GIBCO-BRL, Paisley, UK), and 5 mM D-glucose (Sigma). Properties of H9c2 cells are similar to those in adult cardiomyocytes (5). An interesting feature of this cell line is its ability to differentiate from mononucleated myoblasts to myocyte on reduction of serum concentration. Accompanying myocyte formation is the expression of myogenic transcription factors and calcium channel proteins. During this differentiation process, cells retain several elements of the electrical and hormonal signaling pathway of cardiac cells and have, therefore, become an accepted in vitro model to study the effects of diabetes on the heart (36). To prevent loss of myoblastic properties, cultures were subcultured before they become confluent, and the line recloned periodically with selection for myoblastic cells. In the experiments, 80–85% confluence cells (0.9×10^5 cells/cm^2) were used in serum-depleted media. Hyperglycemia was mimicked by adding D-glucose up to the final concentration of 33 mM. This glucose concentration corresponds to plasma levels of 590 mg/dl. For coincubation studies, recombinant human TGF-β_1, rat interleukin (IL)-10 (20 ng/ml, Peprotech) and/or catalase (50–500 U/ml, Sigma) were added together with the high-glucose medium. Total RNA was studied by quantitative PCR (QPCR).

Histology and immunohistochemistry techniques. Myocardium samples were embedded in paraffin. Four-micrometer paraffin sections were stained with Masson trichrome, Sirius red, and hematoxylin-eosin (H/E) following the manufacturer's instructions (Bio-optical). Cardiac fibrosis was quantified on Sirius red-stained sections with Image pro Plus software. Stained area-to-total area ratio (×100) of an average of 10 fields is indicated in Fig. 1. Cell size was quantified as cell diameter of the average of 50 randomly H/E-stained cells. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to detect apoptosis (CardioTACSTM, R&D Systems). Apoptotic cells presented blue nuclear staining. Negative control was made without dUTP-transferase enzyme (not shown). A semiquantitative estimation of apoptotic cells defined as TUNEL-positive cells was carried out by Metamorph software. Data show the percentage of stained-positive nuclei as average of 10 fields in the myocardium.

For immunohistochemistry (IH), endogenous peroxidase activity was blocked with 3% methanol-H_2O_2. Unspecific sites were treated with a blocking buffer (5% albumin and 10% specific serum in PBS). Primary antibody [anti-collagen I (Calbiochem), anti-CTGF (connective tissue growth factor; Torrey Pines), anti-CD68 or anti-CD3 (for macrophages and T lymphocytes, respectively; Serotec)] was added to the sections in blocking buffer and incubated overnight at 4°C. After washing, secondary biotin-labeled antibody was added, washed, and developed with AB streptavidin-complex and diaminobenzidine chromogen. Infiltrate was quantified with Metamorph software on 10 fields of myocardium. Graph shows the number of CD68 and CD3 positive leukocytes per area (mm^2) of myocardium. In all cases, photographs were taken at ×40 magnification. A scale bar is shown in each photograph.

Protein assays (Western blot, ELISA, and electromobility shift assay). Briefly, a piece of frozen half ventricle was cold pulverized, dissolved in lysis buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 2% SDS + 1/250 protease inhibitors), and vortex for 30 min on ice. After centrifugation at 13,000 rpm, supernatant (total protein extract) was separated and quantified by the bicinchoninic acid method (Pierce). Equal amounts of samples (40 μg) were loaded on an acrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk in Tris-buffered saline + Tween 20 0.05% and incubated with the primary antibody [anti-TGF-β (Abcam), -Fas, -FasL (Fas ligand; Sta. Cruz bt.), -Bax (Pharmingen), -Bcl2 (Sta. Cruz bt.), -cleaved-Asp175-caspase3 (Cell Signalling), -catalase (Calbiochem), -IL-6 (Abcam), -IL-10 (R&D systems), or -TNF-α (Abcam)] overnight at 4°C. Next, membranes were incubated with horseradish peroxidase-labeled secondary antibody and developed with ECL/X-ray films (Amersham). α-Tubulin or GAPDH (Sigma) was the loading control. In Figs. 1–5, we show a representative gel of three experiments of all rats and the semiquantification score (*n*-fold). The protein molecular weights are indicated. Enzyme-linked immunosorbent assay (ELISA; Bender Medsystems) was performed for TGF-β_1 (ref.: BMS623) and IL-10 (ref.: BMS629) in cell culture supernatants. H9c2 were incubated with 33 mM D-glucose for 24–48 h, and media was collected and analyzed following manufacturer's instructions. To block protease activity, media was supplemented with 1% fetal bovine serum.

Electrophoretic mobility shift assay was used to detect nuclear transcription factor activation. Briefly, a frozen piece of ventricle was pulverized, dissolved in lysis buffer (20 mM HEPES, pH 7.5, 20% glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1/250 cocktail protease inhibitors), and vortexed for 30 min on ice. Protein quantification was made as explained above. commercial oligonucleotides [phospho-Smad3/4 (p-Smad3/4) and activator protein-1 (AP-1) from Promega] were labeled with [γ-^{32}P]ATP, incubated 30 min with cellular extracts in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) and separated on acrylamide gels. Gels were run, dried out, and exposed to X-ray films. Protein (nuclear transcription factor) and oligonucleotide binding was semiquantified by densitometry. Competition assay for specific binding was made preincubating the protein extract with the unlabeled or unrelated (not shown) oligonucleotide, before adding the labeled oligonucleotide. In Figs. 1–5, we show a representative gel of three experiments of all rats and the semiquantification score (*n*-fold). The protein-oligonucleotide complexes are indicated. All complexes for each transcription factor were semiquantified together.

QPCR. Analysis of the relative gene expression was performed by retro-transcription and QPCR. Total RNA from left myocardium or cultured cells was dissolved in TRIzol reagent (Invitrogen). After RNA extraction, quality and quantity were measured by absorbance (260- to 280-nm absorbance ratio). Equal amounts of total RNA were reverse transcripted to obtain the cDNA, which was used for multiplex QPCR. The reaction was prepared as follows: for each point, 33 ng of cDNA, 0.25 μl of gene expression assays (0.125 μl target gene + 0.125 μl housekeeping gene), 5 μl premix buffer (polymerase and salts), and RNase free water (up to 10 μl). Applied Biosystems expression assays (Taqman Fam fluorophore) were as follows: angiotensinogen (Ao) (Rn00593114_m1), CTGF (Rn00573960_m1), fibronectin (FN) (Rn00569575_m1), IL-1β (Rn00580432_m1), heme oxygenase 1 (HO-1) (Rn01536933_m1), monocyte chemoattractant protein 1 (MCP-1) (Rn00580555_m1), nitric oxide synthase (NOS)-1 (Rn00583793), and VCAM-1 (Rn00563627_m1). Housekeeping eukaryotic ribosomic 18s was Vic fluorophore (4310893E). Amplification conditions were as follows: 2 min at 50°C, 10 s at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C (Applied B. 7500). All samples were prepared in triplicate to obtain their threshold cycle. If deviation for each triplicate were higher than 0.3 cycles, threshold

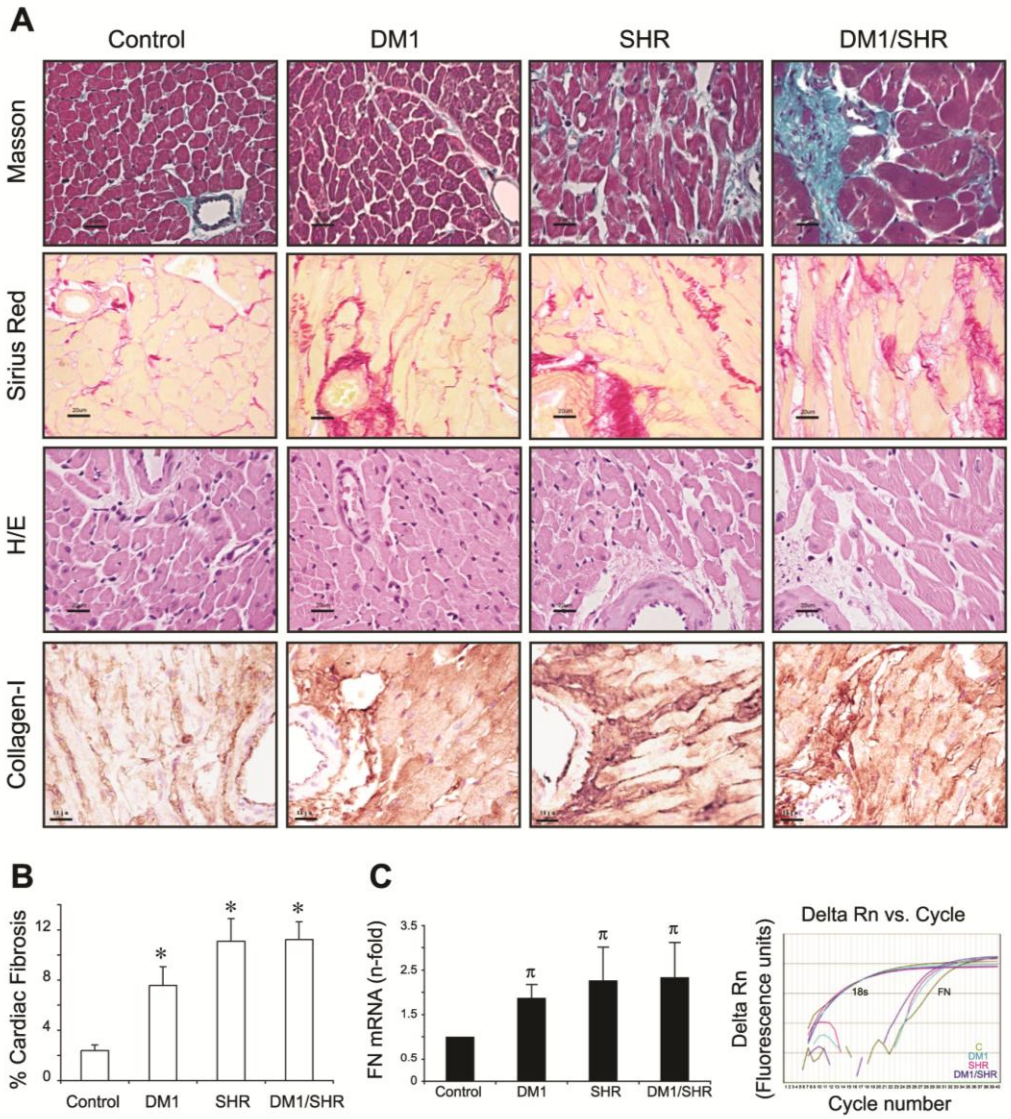


Fig. 1. Morphological changes in the long-term type 1 diabetes mellitus (DM1) and hypertensive myocardium. *A*: Masson trichrome, Sirius red, and hematoxylin-eosin (H/E) staining in streptozotocin (STZ)-induced DM1, spontaneously hypertensive rat (SHR), and DM1/SHR heart. In the *bottom*, type I collagen staining (brown) is shown. *B*: cardiac fibrosis quantification as percentage of red stained area (fibrotic) vs. total area by Sirius red. *C*: fibronectin (FN) mRNA expression. A representative quantitative PCR (QPCR) amplification [delta normalized receptor (Rn) vs. cycle] of a rat of each group (control, DM1, SHR, DM1/SHR) for 18s and FN is also shown. $^{*}P < 0.01$ or $^{\pi}P < 0.05$ vs. control.

cycle was not considered. In each figure, we show a quantification (fold gene vs. 18s) of at least three QPCRs of all rats or cultured cardiomyocytes.

Statistics. Results are expressed as means ± SD. For in vitro experiments, this represented at least three independent experiments. Multiple comparisons were performed by a Kruskal-Wallis test followed by a Mann-Whitney test. A two-tailed $P < 0.05$ was considered significant.

RESULTS

Characterization of the long-term DM1 and hypertensive rat model. STZ is a pancreatic β-cell toxin used to induce chronic and severe hyperglycemia, consistent with experimental DM1 in adult rats (30). In our model, rats were killed 22 wk after DM1 induction. Normotensive STZ-induced DM1 animals presented hyperglycemia (554 ± 28 mg/dl blood glucose) and albuminuria (1,578 ± 314 μg/day) (Supplemental Fig. 1*A*). DM1/SHR rats also showed hyperglycemia (450 ± 65 mg/dl), severe albuminuria (7,804 ± 2,810 μg/day), and high-systolic blood pressure (201 ± 3 mmHg). SHR rats showed higher blood pressure (204 ± 1.8 mmHg) but similar albuminuria than DM1 rats (1,570 ± 147 μg/day). Control rats kept normal blood glucose level (80 ± 2.8 mg/dl), blood pressure (138 ± 11.6 mmHg), and albuminuria (176 ± 4.3 μg/day). As expected, DM1 rats gained significantly less weight compared

with normotensive and SHR rats (Supplemental Fig. 1*A*). In the short-term (6 wk) model, STZ-treated animals presented also hyperglycemia and albuminuria (Supplemental Fig. 1*B*).

Myocardial fibrosis induced by long-term STZ-induced DM1 and hypertension. Masson trichrome, Sirius red, and H/E staining were used to examine the morphological changes in the myocardial interventricular septum. The myocardium from control animals showed normal structure (Fig. 1*A*). However, both DM1 and DM1/SHR rats presented an increased extracellular matrix deposition, as stained by Sirius red (7.5 and 11.2% for DM1 and DM1/SHR, respectively) (Fig. 1, *A* and *B*). Fibrosis was mainly interstitial and perivascular. IH indicated that type I collagen was a main component of the fibrotic extracellular matrix (Fig. 1*A*). In addition, in DM1 and DM1/SHR myocardium, FN mRNA expression was elevated (1.8- and 2.3-fold, respectively, vs. control) (Fig. 1*C*). Interestingly, SHR rats showed similar fibrosis and matrix protein expression as DM1/SHR (Fig. 1). As previously observed (6, 8), extracellular matrix deposition was also detected in the myocardium of short-term (6-wk) STZ-induced DM1 (1.76%) and hypertensive (5.97% for SHR and 2.03% for DM1/SHR) rats vs. control (1.2%) (Fig. 2; Sirius red).

On the other hand, myocardial cell size was increased in long-term DM1 (124.3 ± 1.25% vs. control) and more conspicuously in both SHR and DM1/SHR rats (170.3 ± 1.7% and 208.7 ± 1.87%, respectively. Fig. 1*A*; H/E). A lesser increase in cell size was noted in short-term DM1, SHR, and DM1/SHR (113.77 ± 3.47, 144.0 ± 3.01, and 137.77 ± 2.5%, respectively, Fig. 2; H/E). However, the hypertrophic effect on these pathologies was not an objective of this study.

Long-term DM1 and hypertension promote a proapoptotic milieu in the myocardium. By TUNEL staining, long-standing STZ-induced DM1, SHR, and DM1/SHR rats presented a significant increase of apoptotic cells in the myocardium (Fig. 3*A*). DM1 hearts showed 5.08 ± 1.2% apoptotic nuclei, whereas SHR and DM1/SHR depicted 11.1 ± 2.5 and 14.0 ± 1.5%, respectively ($P < 0.01$ vs. control). As expected, lower apoptotic levels were found in short-term DM1 and hypertensive animals (0.79, 1.63, and 1.22%, respectively. Fig. 2; TUNEL).

The apoptotic mechanisms have not been elucidated in long-term DM1. TNF superfamily proteins, such as FasL promote apoptosis through caspase activation (7, 22). The expression of FasL and its receptor (Fas) was increased in the DM1 (1.43- and 2.3-fold, respectively, vs. control) and DM1/SHR (2.05- and 1.69-fold, respectively, vs. control) myocardium (Fig. 3*B*). Moreover, the Bax (proapoptotic)-to-Bcl2 (antiapoptotic) ratio was higher in the DM1 (7-fold vs. control, Fig. 3*C*, *bottom*) and DM1/SHR myocardium (5.7-fold vs. control). Finally, activation of caspase-3 in DM1 and DM1/SHR was also augmented (3- and 3.5-fold, respectively, vs. control) (Fig. 3*D*). The SHR rats presented

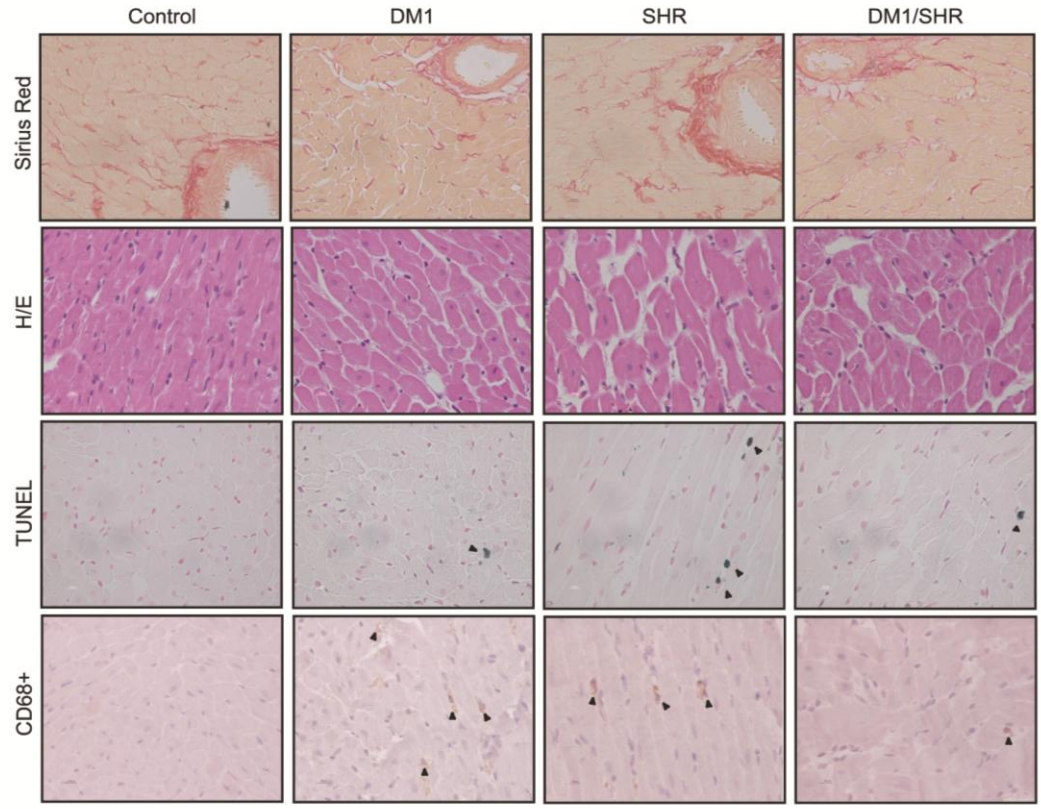


Fig. 2. Short-term STZ-induced DM1 and hypertensive injury in the heart. Fibrosis (by Sirius red), hypertrophy (H/E), apoptosis [terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)], and inflammation (CD68$^+$ detection) representative photographs are shown. Arrowheads indicate apoptotic nuclei or inflammatory cells (macrophages) in each panel.

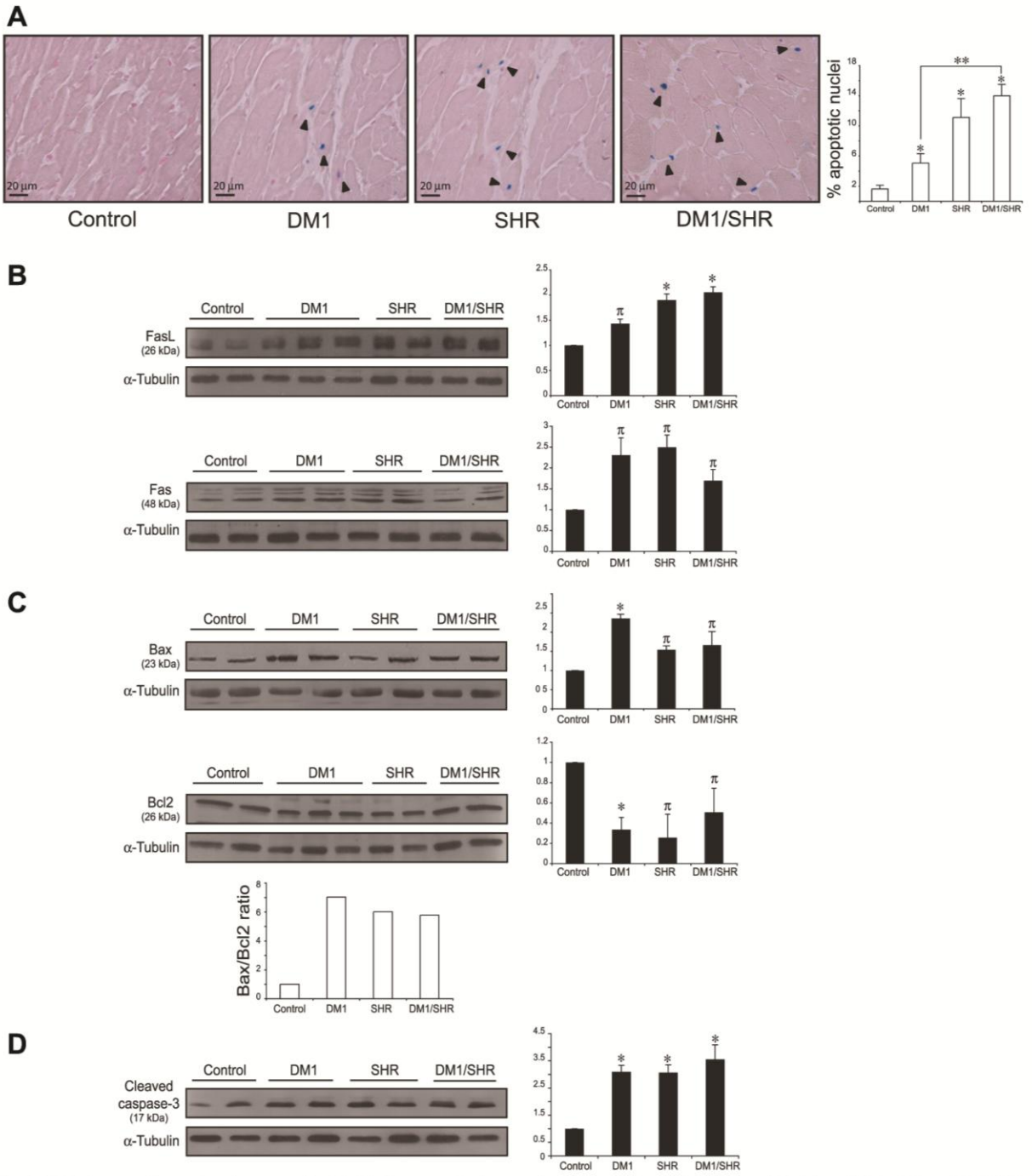
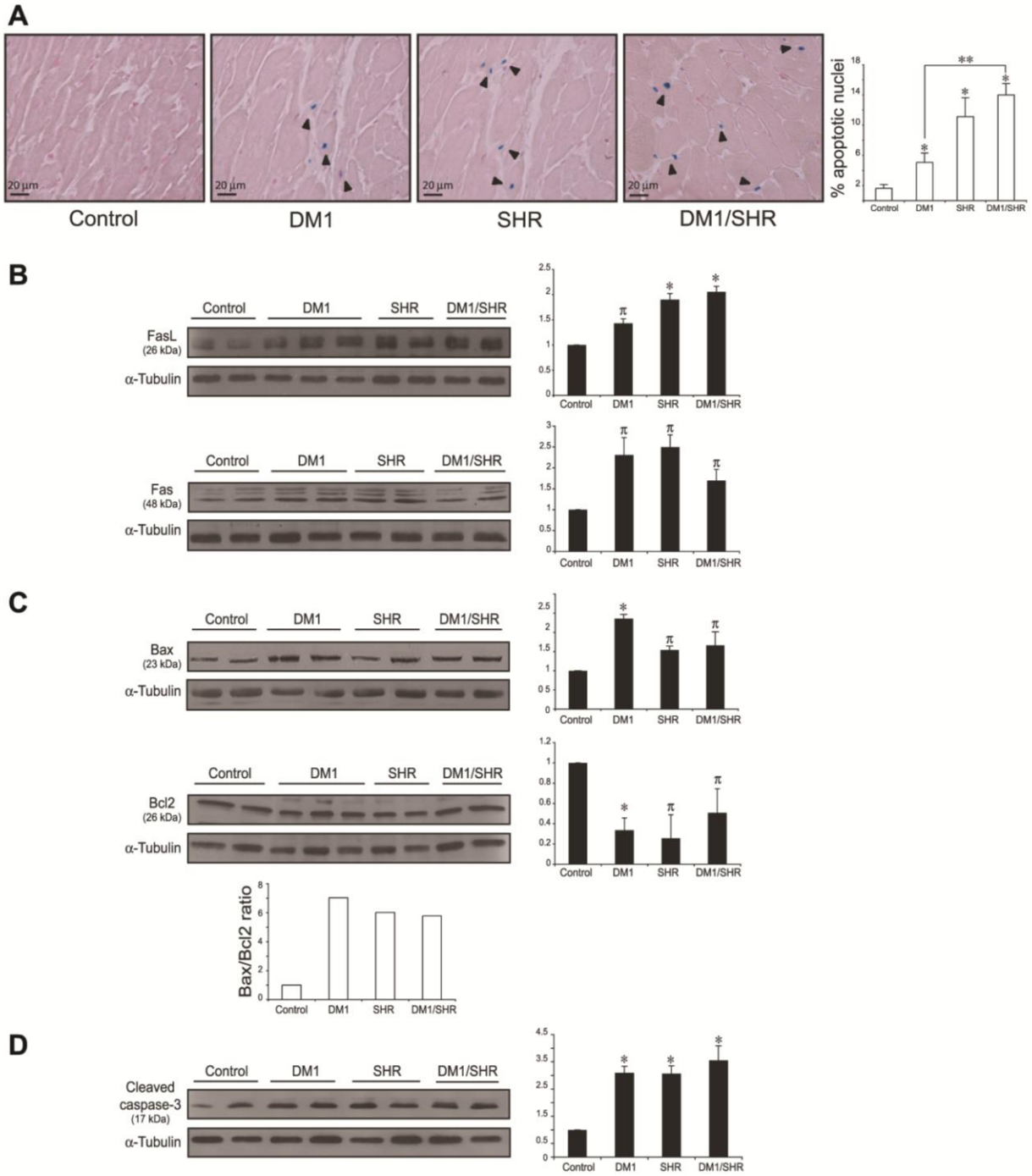


Fig. 3. DM1, hypertension, and cardiac apoptosis. *A*: TUNEL assay stained apoptotic cells (arrow heads) in long-term STZ-induced DM1, SHR, and DM1/SHR myocardium. Semiquantification of apoptosis is showed as percentage of apoptotic nuclei (blue) vs. total nuclei (×100). By Western blot, Fas ligand (FasL) and Fas (*B*); pro- and antiapoptotic Bax, Bcl2, and Bax/Bcl2 ratio (*C*); and caspase-3 (cleaved-activated protein) (*D*) are shown. Semiquantitative scores (*n*-folds) for each protein are also indicated on the *right*. $^{*}P < 0.01$ or $^{\pi}P < 0.05$ vs. control. $^{**}P < 0.01$ vs. DM1.



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similar levels of apoptotic-related factors than DM1/SHR (Fig. 3).

The TGF-β system in long-term DM1 and SHR myocardium. The TGF-β system is a key mediator of fibrosis in the heart and other tissues through deposition of extracellular matrix proteins, such as FN and type I collagen, from local cells (13a, 26, 34). In addition, TGF-β may increase the expression of lethal molecules, such as Fas (23). In long-term STZ-induced DM1 and SHR myocardium, TGF-β_1 protein levels were augmented (3.2 and 3.3-fold, respectively, vs. control, Fig. 4*A*). When DM1 was associated with hypertension, there was a further increase in TGF-β_1 levels (4.8-fold, $P < 0.01$ vs. control, $P < 0.05$ vs. SHR and $P =$ nonsignificant vs. DM1).

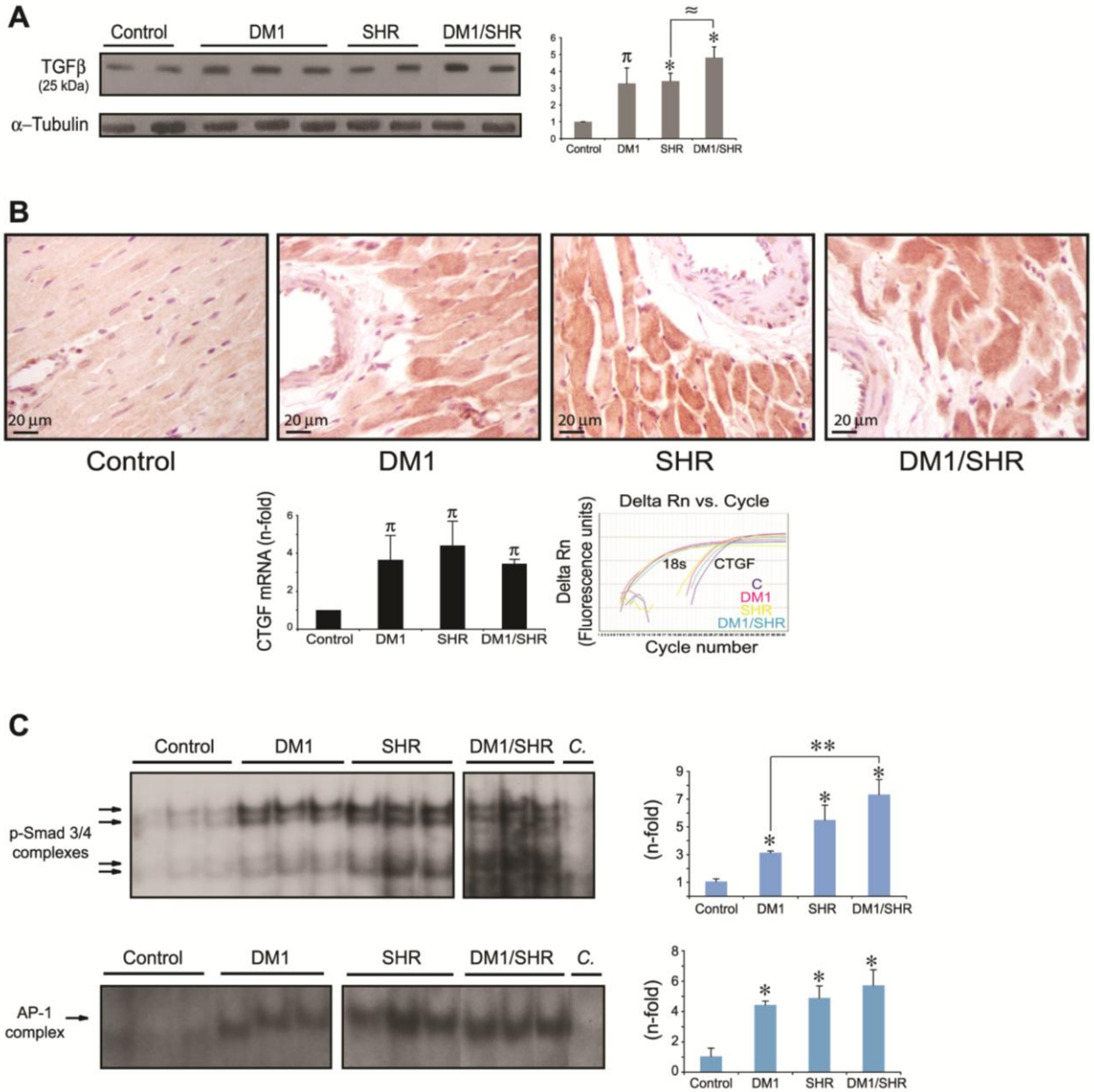


Fig. 4. Long-term DM1, hypertension, and myocardial transforming growth factor (TGF)-β system. *A*: myocardial TGF-β protein levels by Western blot. Semiquantification (*n*-folds) for each protein is also shown (*right*). *B*: connective tissue growth factor (CTGF) myocardial staining and mRNA expression. A representative QPCR amplification (delta Rn vs. cycle) of a rat of each group (control, DM1, SHR, DM1/SHR) for 18s and CTGF is also shown. *C*: by EMSA, TGF-β-linked phosphor-Smad3/4 (p-Smad3/4), and activator protein (AP)-1 activated complexes (arrows) in STZ-induced DM1, SHR, and DM1/SHR myocardium. Semiquantification for each transcription factor is shown (*right*). C, means specificity competition assay (unlabeled oligonucleotide). Nonspecific probe was similar for all rats (not shown). $^{*}P < 0.01$ or $^{\pi}P < 0.05$ vs. control, $^{\sim}P < 0.05$ vs. SHR, and $^{**}P < 0.01$ vs. DM1.

CTGF is a well-known mediator of the TGF-β system actions (17, 32), although its expression has not been described in DM. CTGF expression was increased (mainly in myocytes) in long-standing DM1, SHR, and in DM1/SHR myocardium (IH and QPCR; 3.6-, 4.2-, and 3.5-fold, respectively, vs. control mRNA expression, Fig. 4*B*).

We also studied the activation of TGF-β-linked transcription factors p-Smads and AP-1. Using specific oligonucleotides for both Smad3/4 (against p-Smad3 and -Smad4 members) and AP-1, we detected activated transcription complexes (Fig. 4*C*, arrows). Four p-Smads complexes and one AP-1 complex were observed in the myocardium. Competition assay with cold oligonucleotides demonstrated the specificity of the binding (Fig. 4*C*, C). In long-term STZ-induced DM1 and SHR myocardium, all four p-Smads complexes were increased (2.9- and 5.2-fold, respectively, $P < 0.01$ vs. control). Added hypertension enhanced the DM1 effect (7.5-fold, $P < 0.01$ vs. DM1 and control). AP-1 was also activated in the three groups (4.2-, 4.6-, and 5.4-fold, respectively, $P < 0.01$ vs. control).

Inflammation in long-term DM1 and SHR myocardium. The presence of myocardial inflammation has been previously described in SHR (19, 27) and short-term STZ-induced DM1 (38). We now confirmed these data in short-term DM1 rats by CD68 staining (for macrophage detection). At this stage, DM1, SHR, and DM1/SHR myocardium showed inflammatory cell recruitment (3.77, 3.58, and 1.88 cells/mm^2, respectively) (arrowheads, Fig. 2; CD68$^+$). However, in the long-standing model, we did not observe significant CD68 or CD3 positive cells (for T lymphocytes) infiltrating cells, both in DM1 and DM1/SHR myocardium (Fig. 5*A*). Increased expression of proinflammatory cytokines IL-1β and TNF-α was described in short-term myocardial injury (38). In our long-term DM1 model, IL-1β, TNF-α, MCP-1, IL-6, and VCAM-1 were not significantly increased in DM1 hearts (Fig. 5*B*). However, we confirmed increased expression of TNF-α in short-term DM1 heart (1.92-fold vs. control, Fig. 5*C*). TNF-α was also induced in both long- and short-term SHR (2.05- and 1.61-fold, respectively, vs. control; Fig. 5*C*) and short-term DM1/SHR myocardium (1.65-fold vs. control, Fig. 5*C*).

In addition, proinflammatory factor NF-κB, which regulates the expression of many of these genes, was not activated in long-term DM1 (Fig. 5*D*). In addition, DM1 showed a dominant effect on NF-κB activation, since its presence attenuated the hypertensive effects (Fig. 5*D*).

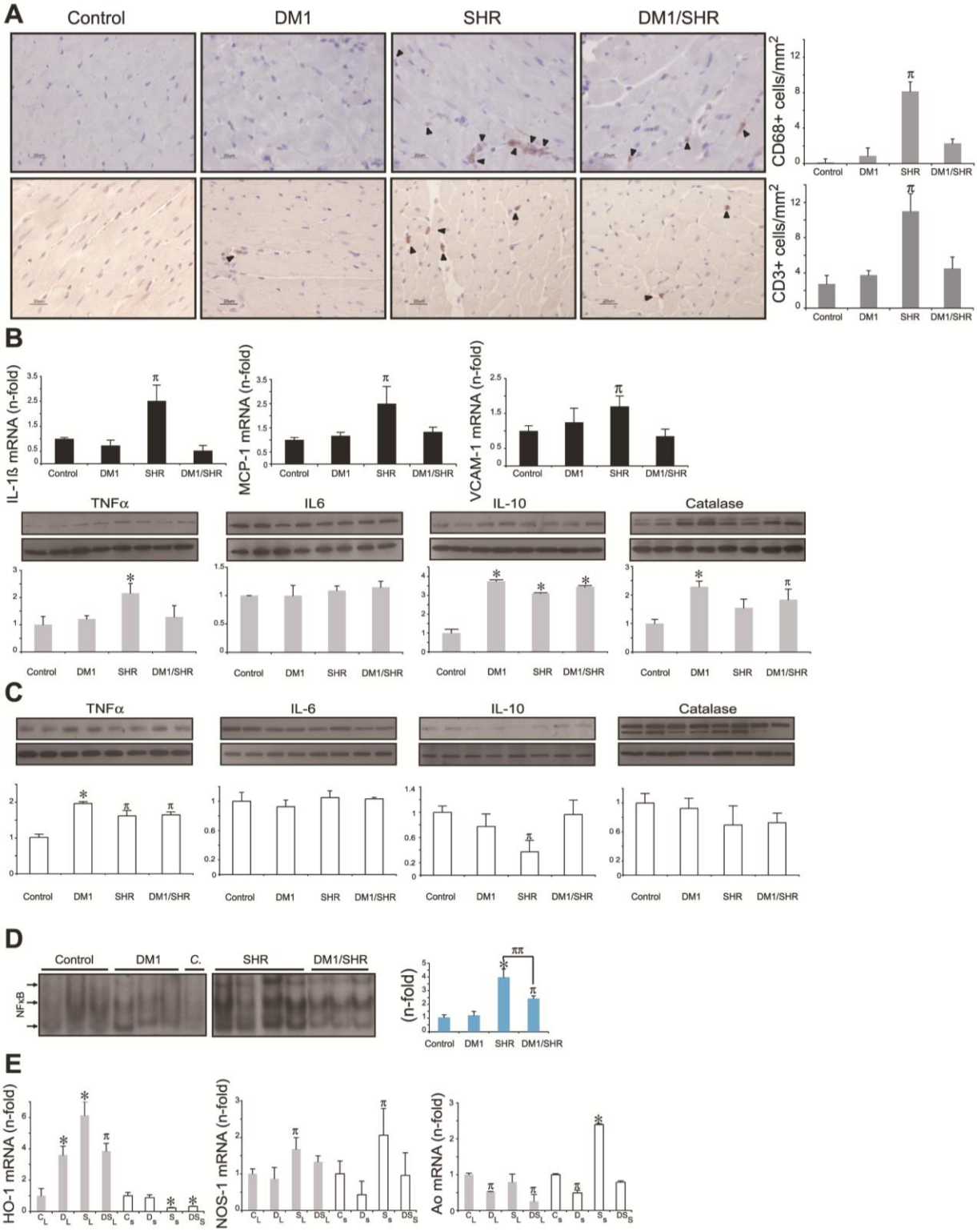
Anti-inflammatory cytokines and antioxidants in experimental DM1 heart and glucose-incubated cardiomyocytes. We examined the expression of anti-inflammatory and antioxidant molecules in long-term DM1 myocardium. IL-10 was stimulated in long-term DM1 (3.73-fold vs. control), but not in short-term DM1 (Fig. 5, *B* and *C*). The anti-oxidants catalase (Fig. 5, *B* and *C*) and HO-1 (Fig. 5*E*) presented a similar induction only in long-term DM1 (2.31- and 3.59-fold, respectively, vs. control). Accordingly, the prooxidant neuronal NOS-1 was not elevated in the DM1 heart (Fig. 5*E*). Long-term SHR hearts presented increased IL-10 (2.97-fold vs. control) and HO-1 (6.13-fold vs. control), unchanged levels of catalase, and elevated NOS-1 (1.67-fold vs. control) (Fig. 5, *B* and *E*). In short-term SHR myocardium, IL-10, catalase, and HO-1 were decreased or unchanged (Fig. 5, *C* and *E*), whereas NOS-1 was stimulated (Fig. 5*E*). All of these effects were attenuated by the coexistence of both pathologies in DM1/SHR rats (Fig. 5, *B*, *C*, and *E*).

In addition, we evaluated the presence of a local activated RAA system. The expression of the angiotensin II precursor, Ao, was reduced in both long- and short-term DM1 (Fig. 5*E*). In contrast, Ao was enhanced in short-term SHR rats (1.62-fold vs. control), and this response disappeared again in DM1/SHR hearts.

In vivo findings suggest that long-term DM1 may activate secondary mediators, which dampen the myocardial inflammatory mechanism, and diminished proinflammatory responses to hyperglycemia. We next studied whether cultured cardiomyocytes could secrete anti-inflammatory and antioxidant molecules when exposed to long incubation of high-glucose medium. By ELISA, we detected both TGF-β and IL-10 molecules in H9c2 media after 48 h of high-glucose incubation (Fig. 6*A*). The increase in IL-10 secretion lagged behind that of TGF-β. Thus we analyzed the direct effect of exogenous TGF-β and IL-10 on high-glucose-induced proinflammatory responses. High glucose induced also proinflammatory MCP-1 (5 min to 1 h) and VCAM-1 (5 min to 6 h) expression in cardiomyocytes (Fig. 6*B*). The maximal MCP-1 expression induced by high glucose was significantly decreased by coincubation with anti-inflammatory TGF-β or IL-10 (0.33- and 0.35-fold, respectively vs. high glucose alone at 30 min). Both TGF-β and IL-10 also reduced VCAM-1 expression induced by high glucose (0.22- and 0.49-fold, respectively, vs. 3-h high glucose alone). Incubation with both TGF-β and IL-10 in high-glucose medium abolished MCP-1 peak expression. VCAM-1 mRNA was similarly reduced (Fig. 6*B*). Finally, catalase was also added to the high-glucose media to the cardiomyocytes. After 15–30 min of incubation, catalase (150–300 U/ml) normalized MCP-1 and VCAM-1 glucose-induced expression in a similar manner than the anti-inflammatory cytokines (0.43- and 0.4-fold, respectively, vs. high-glucose alone).

DISCUSSION

Fibrosis and apoptosis are important events in the DM heart. Both responses have been described in human (13, 21, 35) and short-term experimental DM1 (our data, Refs. 2, 8, 9, 37, 38) and hypertension (19, 27). We now confirm that fibrosis and apoptosis are also features of long-term STZ-induced DM1 and hypertension. TGF-β_1 can be a primary mediator for both fibrosis and apoptosis (13a, 23, 34). Via AP-1 activation, TGF-β is involved in cardiac hypertrophic growth, apoptosis, and fibrosis (34). Through Smads proteins, TGF-β_1 also increases extracellular matrix deposition by induction of ROS and calcium influx (34). TGF-β activates caspases and the Fas pathway (23), and TGF-β_1-induced CTGF leads to cancer cell apoptosis through a diminution of Bcl2 expression (17). In both short- and long-term DM1, TGF-β_1 overexpression has been described (3, 37). We have observed increased TGF-β expression in long-standing experimental DM1 in association with an increased profibrotic (collagen I, FN, CTGF) and proapoptotic (Bax and Fas) factors and enhanced activity of TGFβ-linked transcription factors (AP-1 and p-SMADs). Thus activation of the TGF-β pathway may underlie the extracellular matrix deposition and apoptosis in the long-term DM1 and hypertensive myocardium. In addition, hypertrophy was also stimulated in these hearts and could be linked to the observed cell apoptosis (and matrix deposition). It is noteworthy that


A
Control
DM1
SHR
DM1/SHR
CD68+ cells/mm2
CD3+ cells/mm2
B
IL-1ß mRNA (n-fold)
MCP-1 mRNA (n-fold)
VCAM-1 mRNA (n-fold)
TNFα
IL6
IL-10
Catalase
C
TNFα
IL-6
IL-10
Catalase
D
Control
DM1
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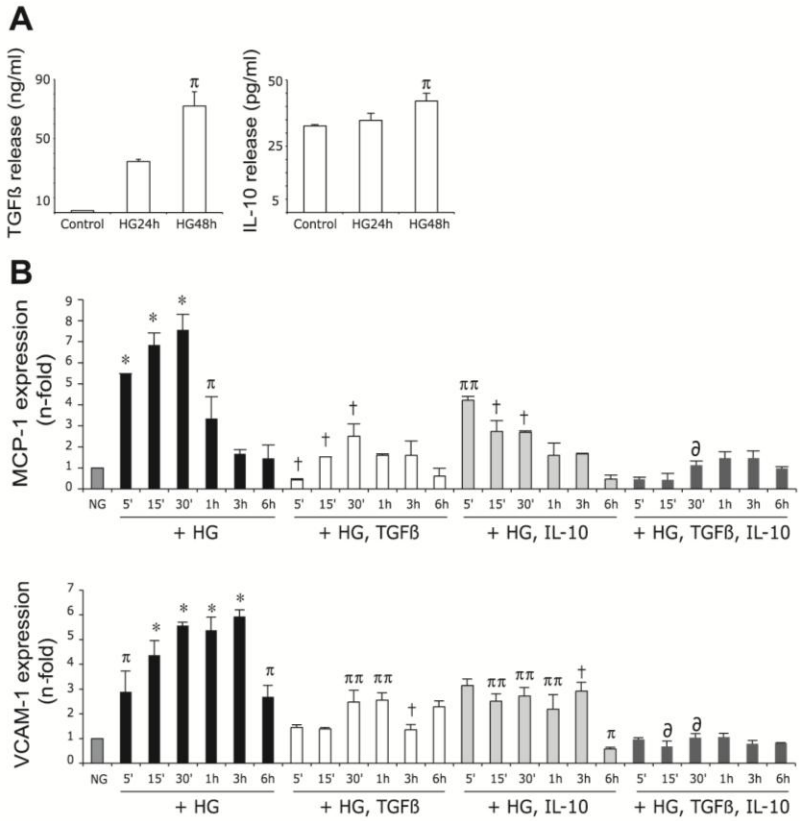

Fig. 6. Anti-inflammatory proteins blunt glucose-induced proinflammatory genes in cultured cells. *A*: H9c2 cardiomyocytes release TGF-β_1 and IL-10 to the media after high-glucose (HG) incubation ($^{\pi}P < 0.05$ vs. control). *B*: H9c2 were incubated with normal (NG) or HG medium. Some cells were coincubated with HG medium and TGF-β, IL-10, or both together. MCP-1 (*top*) and VCAM-1 (*bottom*) mRNA expressions were quantified by QPCR. $^{*}P < 0.01$ or $^{\pi}P < 0.05$ vs. control; $^{\dagger}P < 0.01$ or $^{\pi\pi}P < 0.05$ vs. HG alone; and $^{\partial}P < 0.05$ vs. HG + TGF-β and HG + IL-10 alone.

TGF-β is also an anti-inflammatory cytokine (22) and may be involved in the control of cardiac inflammation.

In human studies, nonspecific serum inflammatory markers, such as IL-6, TNF-α, troponin, or C-reactive protein, suggesting systemic inflammation, have been linked to cardiovascular dysfunction in diabetic patients (25, 30, 33). However, there is a lack of information showing local inflammatory leukocytes on human myocardial biopsies or necropsies. In this sense, our results demonstrated that long-standing STZ-induced DM1 and SHR cardiomyopathies differed in a key feature. Long-term DM1 myocardium did not present inflammatory cells, while the inflammatory infiltrate, expression of inflammatory mediators (TNF-α, IL-1β, MCP-1, and VCAM-1) and activation of the proinflammatory transcription factor NF-κB were prominent in long-term SHR heart injury. However, increased TNF-α expression and macrophage infiltration were noted in short-term DM1 heart injury. These results are in agreement with a recent work, which shows unchanged IL-6, VCAM-1, and MCP-1 expression in the rat myocardium from 7 to 24 wk after type 2 diabetes (11).

In cardiomyocytes, we also show that a high-glucose environment induces the release of TGF-β_1, which can modulate proinflammatory gene expression. Nevertheless, it is unlikely that TGF-β_1 is the only factor suppressing inflammation in the long-term STZ-induced DM1 heart, since its expression and actions (such as p-Smad and CTGF activation) are increased also in hypertensive cardiomyopathy. In the heart, both cardiomyocyte and noncardiomyocyte cells can express anti-inflammatory IL-10 (40), which is increased in spleen and heart, but not in liver, thymus, or bone of diabetic rats (24). Opposite to short-term DM1, we have detected an increased expression of IL-10 in long-standing DM1 myocardium (parallel to a unchanged TNF-α and IL-6 expression). In long-term SHR hearts, although IL-10 was also elevated, there was overexpression of proinflammatory TNF-α. In addition, IL-10 was also released from cultured cardiomyocytes after high-glucose stimulation. In this sense, in DM1 patients, blood mononuclear cells released more IL-10 than controls, and this was associated with a decrease of proinflammatory IL-1, IL-6, and TNF-α secretion (12). In our cell cultured experiments, IL-10 secretion

Fig. 5. Myocardial inflammation in STZ-induced DM1 and hypertensive hearts. *A*: infiltrating $CD68^+$ (macrophages) and $CD3^+$ (T-lymphocytes) cells in long-term rat myocardium (arrowheads). Quantification is indicated. *B*: by QPCR, proinflammatory [IL-1β, monocyte chemoattractant protein (MCP)-1, and VCAM-1] gene expression in long-standing DM1 and hypertensive rats are shown. By Western blot, TNF-α, IL-6, IL-10, and catalase levels in long-term DM1 and hypertensive myocardium are shown. *C*: TNF-α, IL-6, IL-10, and catalase were also examined in the short-term model. *D*: by EMSA, proinflammatory transcription factor NF-κB activation in long-term model. C, means specificity competition assay (unlabeled NF-κB). Nonspecific probe was similar for all rats (not shown). Semiquantitative score is indicated. *E*: anti-oxidant [heme oxygenase-1 (HO-1)] and oxidant [nitric oxide synthase-1 (NOS-1) and angiotensinogen (Ao)] gene expression in both long and short DM1, SHR, and DM1/SHR rats. C_L, D_L, S_L, and DS_L: long control, DM1, SHR, and DM1/SHR rats, respectively; C_S, D_S, S_S, and DS_S: short control, DM1, SHR, and DM1/SHR rats, respectively. $^{*}P < 0.01$ or $^{\pi}P < 0.05$ vs. control, and $^{\pi\pi}P < 0.05$ vs. SHR.

was delayed in a temporal manner with respect to TGF-β_1. Indeed, exogenous IL-10 decreased the inflammatory response induced by high-glucose media, and this effect was further enhanced by TGF-β_1. These data are also consistent with the observation that, in cardiomyocytes, IL-10 antagonizes IL-1β and IL-6 expression, ROS production, and inflammation promoted by TNF-α (4, 20). Thus IL-10 may contribute to the absence of inflammation observed in long-term DM1 heart. In this sense, IL-10 has been classified as a protective interleukin in the cardiovascular system (10). Decreased IL-10 plasma levels are associated with a high incidence of cardiovascular events (16). Moreover, systemic induction of IL-10 expression in Dahl salt-sensitive rats reduced inflammatory infiltration, hypertrophy, and cardiac dysfunction in the hypertensive heart (27).

The oxidative state may play a role in the cardiac inflammation related to DM1. Activation of RAA system during DM1 is associated with increased oxidative damage and cardiomyocyte death, which contributes to the increased interstitial fibrosis and inflammation (2, 6, 9). Blockade of the RAA system in STZ-treated rats attenuates cardiac dysfunction, partially through the reduction of ROS production (6, 25, 37). Importantly, the change in the expression of RAA system components in DM1 hearts appears to be local and independent of the circulating RAA system. Thus STZ-induced DM1 heart apoptosis peaked at 3 days and decreased after 28 days, in correlation with a reduction in RAA system components (8, 14). In this sense, and in contrast to hypertension, we have observed a decrease in Ao and unchanged NOS-1 expression in long-term DM1 hearts. In parallel, antioxidants catalase and HO-1 were overexpressed. In cardiomyocytes, catalase also decreased proinflammatory glucose-induced genes. Thus, together with the increase of the anti-inflammatory-to-proinflammatory ratio, long-term DM1 heart may develop compensating mechanisms to dampen myocardial damage. Nevertheless, more experimental models targeting anti-inflammatory and antioxidant molecules are needed to fully confirm their potential therapeutic role in DM cardiac disease. In this regard, Ao expression is not always directly associated to increase RAA system activity and NOS-1 may inhibit xanthine oxidoreductase, which is responsible for ROS production (2a).

Finally, the concurrence of both DM1 and hypertension pathologies worsens the deleterious effect of either disease alone and accelerates cardiovascular mortality in humans (13, 21). However, at short- and long-term experimental stages, there was not a consistent additive effect when both disorders are experimentally combined. SHR and DM1/SHR rats showed similar fibrosis, apoptosis, and expression of related proteins. More interestingly, in our work, the presence of DM1 dampened the inflammatory changes observed in SHR animals. A concordant previous report showed that 1-wk STZ treatment does not further increase myocardial contractile dysfunction, oxidative state, and apoptosis in SHR rats (9).

Study limitations. An echocardiographic examination would have been useful to provide a functional correlation of the histological and molecular abnormalities found. In this sense, it has been demonstrated previously in rats that both systolic and diastolic dysfunctions are evident after 6 wk of DM1 (38). Our long-term rats were analyzed after 22 wk of DM1.

Conclusions. We demonstrated that long-standing STZ-induced DM1 is characterized by fibrosis and apoptosis in the heart, in association with the activation of the TGF-β system. These effects were independent of hypertension and not enhanced by its presence. Long-standing DM1 cardiomyopathy differs also from early DM1 and long-term hypertensive myocardial injuries by the absence of an inflammatory response. Anti-inflammatory molecules expressed in the DM1 heart (and by high-glucose exposed cardiomyocytes), such as IL-10 and TGF-β, together with reduced local TNF-α, RAA/nitric oxide systems modulation, and antioxidant production, may account for this finding. Further investigations should address the therapeutic consequences of these differences in the treatment of DM1 and hypertensive heart damage.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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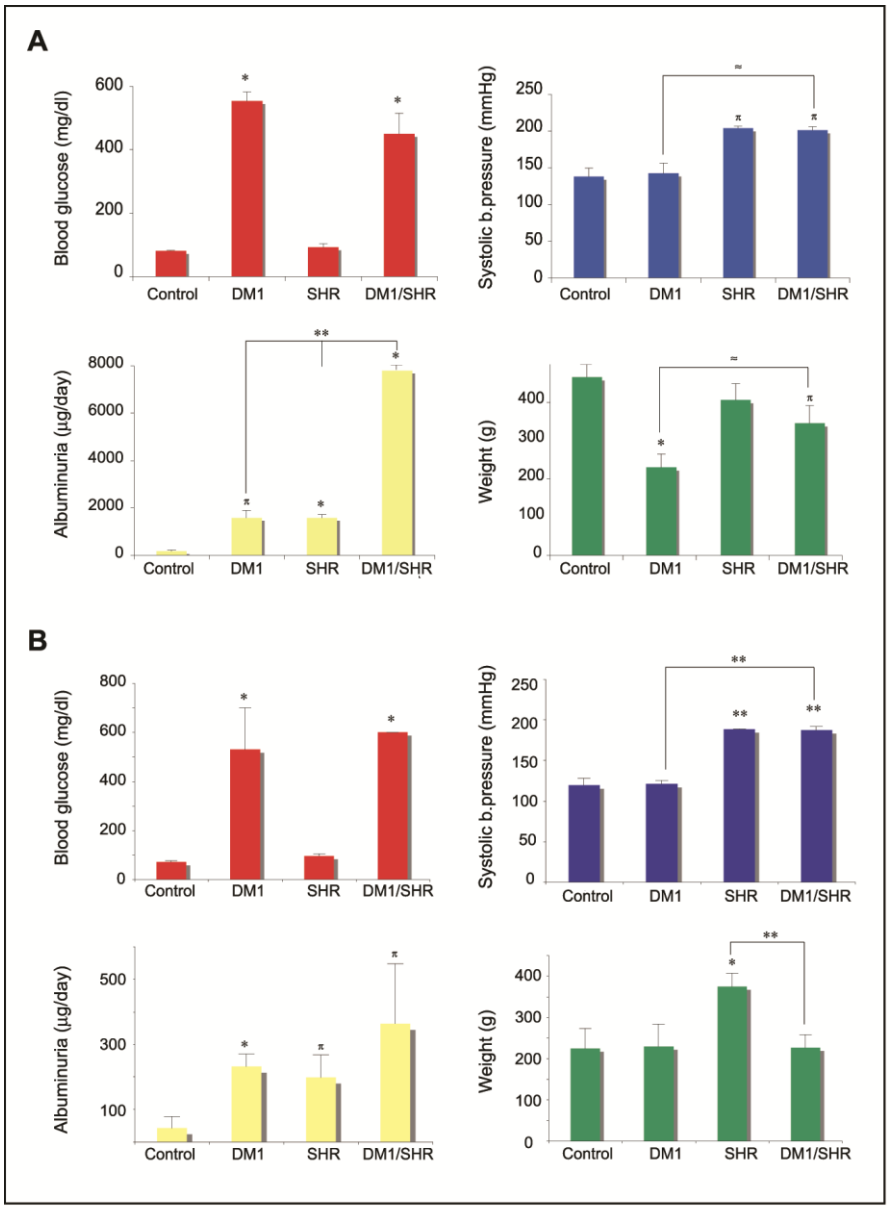

Figure Suppl. 1.

Figure legends

Figure Suppl. 1. Characterization of the long and short-term DM1 model in rat. After 22 **(A)** or 6 weeks **(B)** STZ-treatment, glycemia, systolic blood pressure and albuminuria levels were measured. Rats weight is also shown. * $p<0.01$ or π $p<0.05$ vs. control, and ** $p<0.01$ or $\approx$ $p<0.05$ vs. DM1 or SHR.

2. EXPRESIÓN PROTEÓMICA DIFERENCIAL EN EL CORAZÓN DIABÉTICO E HIPERTENSO. PAPEL DE PPARα EN LA HIPERTROFIA CARDIACA ASOCIADA.

Pocos estudios han analizado comparativamente los efectos sobre el corazón de la DM1 crónica y la hipertensión, así como sus efectos combinados. Las técnicas de proteómica permiten el análisis comparativo de la expresión de cientos de proteínas de diferentes muestras biológicas al mismo tiempo[174]. Los datos resultantes pueden ser incluidos *a posteriori* en estudios bioinformáticos de predicción de rutas moleculares. Otros autores han demostrado por estudios proteómicos la alteración de proteínas cardiacas del citoesqueleto, metabolismo y apoptosis en estadios agudos y crónicos de DM1[77,86,175] e hipertensión[67,68,119]. Concretamente, las enzimas de β-oxidación y moléculas pro-hipertróficas y apoptóticas estaban desreguladas. En nuestros resultados, en estadios crónicos de DM1, hipertensión y DM1 con hipertensión asociada se encontraron 24, 53 y 53 proteínas alteradas, respectivamente. El miocardio DM1 presentaba sobreexpresión de proteínas pro-apoptóticas y del citoesqueleto, y disminución de proteínas anti-apoptóticas y enzimas metabólicas mitocondriales. Tanto en rata hipertensa como en DM1-hipertensa estos cambios estaban acrecentados presentando, además, un descenso en las enzimas de la β-oxidación. Los corazones DM1-hipertensos mostraron además modificaciones en factores pro-hipertróficos y anti-apoptóticos específicos, y en enzimas mitocondriales de la cadena transportadora de electrones. Tras la confirmación de estos niveles por técnicas bioquímicas, las proteínas fueron clasificadas en distintas vías biológicas mediante análisis bioinformáticos. Estos estudios sugirieron la implicación de PPAR, y moléculas pro-hipertróficas. En este sentido, en cardiomiocitos en cultivo sometidos a altas dosis de glucosa y AngII, la estimulación de PPARs, concretamente de PPARα, redujo la hipertrofia y la expresión de factores pro-hipertróficos, como ANXA5. Previamente se había visto que el tratamiento con agonistas PPARα mejoraba la apoptosis y disfunción cardiaca en la miocardiopatía diabética experimental[54,15]. La activación de PPARα, podría reflejar una respuesta compensatoria frente a la alteración del metabolismo, apoptosis e hipertrofia en la MCD o hipertensión.

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Proteome changes in the myocardium of experimental chronic diabetes and hypertension
Role of PPARα in the associated hypertrophy

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ABSTRACT

Diabetes with or without the presence of hypertension damages the heart. However, there is currently a lack of information about these associated pathologies and the alteration of linked proteins. For these reasons, we were interested in the potential synergistic interaction of diabetes and hypertension in the heart, focusing on the proteome characterization of the pathological phenotypes and the associated hypertrophic response. We treated normotensive and spontaneously hypertensive (SHR) rats with either streptozotocin or vehicle. After 22 weeks, type-I diabetic (DM1), SHR, SHR/DM1 and control left-ventricles were studied using proteomic approaches. Proteomics revealed that long-term DM1, SHR and SHR/DM1 rats exhibited 24, 53 and 53 altered proteins in the myocardia, respectively. DM1 myocardium showed over-expression of apoptotic and cytoskeleton proteins, and down-regulation of anti-apoptotic and mitochondrial metabolic enzymes. In both SHR and SHR/DM1 these changes were exacerbated and free fatty-acid (FFA) ß-oxidation enzymes were additionally decreased. Furthermore, SHR/DM1 hearts exhibited a misbalance of specific pro-hypertrophic, anti-apoptotic and mitochondrial ATP-carrier factors, which could cause additional damage. Differential proteins were validated and then clustered into different biological pathways using bioinformatics. These studies suggested the implication of FFA-nuclear receptors and hypertrophic factors in these pathologies. Although key ß-oxidation enzymes were not stimulated in DM1 and hypertensive hearts, peroxisome proliferator-activated receptors-α (PPARα) were potentially activated for other responses. In this regard, PPARα stimulation reduced hypertrophy and pro-hypertrophic factors such as annexin-V in high-glucose and angiotensin-II induced cardiomyocytes. Thus, activation of PPARα could reflect a compensatory response to the metabolic-shifted, apoptotic and hypertrophic status of the hypertensive-diabetic cardiomyopathy.

Abbreviations: DM1, type-I diabetic; STZ, Streptozotocin; SHR, Spontaneously Hypertensive Rats; FFA, Fatty Acid; ROS, Reactive Oxygen Species; PPAR, Peroxisome proliferator-activated receptors; HNF4α, Hepatocyte Nuclear Factor-4α; PGC1α, PPARγ coactivator-1α; MEF2, Myocyte-enhancing factor-2; ERRα, Estrogen-related receptor-α; CPT1b, Carnitine palmitoyltransferase 1B; ACADL, Acyl- Coenzyme A dehydrogenase,long chain; ACADM, Acyl-Coenzyme A dehydrogenase,medium chain; PDK4, Pyruvate dehydrogenase kinase-4; DIGE, Differential In-Gel Electrophoresis; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time Of Flight; MS, Mass Spectrometry.

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1. Introduction

Diabetes is currently a worldwide pandemic, which is expected to worsen in the coming years. More than 36 million people will have type-I diabetes by 2030, two thirds of whom may die because of cardiovascular complications [1]. In humans, cardiac damage can be directly induced by both type-I and type-II diabetes and exacerbated by the frequent coexistence of hypertension [2]. However, few studies have analysed the combined and comparative effects of chronic diabetes and hypertension on the heart. Knowledge of the altered proteomes linked to specific cellular mechanisms present in the pathological phenotypes may provide key information for the field.

Hyperglycemia alters metabolic, structural and contractile proteins activating cellular responses such as hypertrophy and apoptosis in the heart [1–3]. These same responses are also seen in hypertensive cardiomyopathy [4,5]. We previously described cardiac hypertrophy and apoptosis by using an experimental model of long-term DM1 and hypertension [3]. According to these studies, the combined effect of type-I diabetes and hypertension did not increase the fibrotic and inflammatory rates in the rats. However, given that hypertrophy and apoptosis were further stimulated when both pathologies coexisted, it follows that key information may be obtained by identifying altered hypertrophic/apoptotic proteins and related pathways in the diabetic heart, and whether or not it occurs when diabetes associated with hypertension. Proteomics allows the comparative expression analysis of hundreds of proteins in different biological samples at the same time [6]. The resulting data can be also included as input for bioinformatics studies carried out to predict molecular pathways. In previous data, proteomic studies revealed alteration of the cytoskeleton, metabolic and apoptotic proteins in short-term DM1 [7–11] and both short- and long-standing hypertension [12–14]. In particular, free fatty-acid (FFA) oxidation enzymes and pro-hypertrophic/apoptotic molecules were dysregulated. The peroxisome proliferator activated receptors (PPARs) are members of a nuclear receptor family of transcription factors. PPARα is the most abundant PPAR-isoform in the heart and FFA are putative endogenous ligands for these receptors. Under ligand stimulation, PPARα recruits specific co-activators (such as PGC1α) and other transcription factors to regulate fuel homeostasis genes, among others [15]. Impairment of FFA oxidation is a significant characteristic of cardiac hypertrophy [16], whose attenuation may be a promising therapeutic target for the prevention of cardiac apoptosis and failure [17]. In experimental diabetic cardiomyopathy, a PPARα-agonist treatment ameliorated the induced cardiac apoptosis and dysfunction [18,19]. In this work we have investigated the putative synergistic proteomes of chronic diabetes and hypertension in the heart, and the potential beneficial role of PPARα activation in the associated hypertrophy.

2. Methods

2.1. *Long-term hypertensive-diabetic model in rats; study design*

This work is a continuation of a previous study performed using an experimental model of chronic diabetes and hypertension in rats [3]. Six-week-old spontaneously hypertensive rats (SHR) and normotensive (Wistar) male rats as control, received either three streptozotocin injections (50 mg/Kg/day) or vehicle. As a result, there were four different groups: normotensive type-I diabetic (DM1), SHR, SHR/DM1 and Wistar rats. Blood glucose was monitored twice a week (10 a.m.) using a glucometer. Then, where needed, basic insulin (Insulatard NPH) was administrated (by intramuscular injection) in diabetic rats to prevent severe hyperglycemia, while maintaining the blood glucose between 400–600 mg/dl. 3 IU insulin were injected when blood glucose raised to 300–450 mg/dl, and 4 IU insulin when blood glucose reached 450–600 mg/dl. After 22 weeks, the rats were anesthetized (2.5% isofluorane) and the heart isolated. These investigations adhered to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and approval for these experiments was granted by the Ethics Committee of the hospital.

In this work we have used frozen pieces and paraffin slices of left ventricles to carry out proteomic, biochemical and histological studies. To determine the proteomic pattern, samples from DM1, SHR, SHR/DM1 and control rats were analysed using proteomic approaches (DIGE analysis; n=6, each group). Then, to predict related cellular pathways, the resulting lists of differentiated proteins were used as inputs in bioinformatics software for molecular prediction (IPA®, Pathway Architect®). To confirm protein implication, the expression/activation of key differentiated proteins and suggested factors involved mainly in hypertrophy were validated by histological (immunohistochemistry) and biochemical (Western B., EMSA) experiments in myocardium samples (n=6-8, each group). Next, to further investigate into the stimulated hypertrophic response, cultured cardiomyocytes (n≥3, independent experiments) were used for the characterisation of specific mediators (immunocytochemistry, Western B., QPCR).

2.2. *Differential in-gel electrophoresis (DIGE) and predictive analysis*

Left ventricle samples from DM1, SHR, SHR/DM1 and control rats (n=6, each group) were powdered and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris–pH 8.5) containing 50 mM DTT and additionally broken with the 2D Grinding Kit (GE Healthcare, Uppsala, Sweden), and the proteins were labeled with fluorescent dyes for DIGE analysis (see supplemental methods), as described previously [20]. After electrophoresis, the gels were scanned with a Typhoon 9400 scanner (GE Healthcare) at 100 μm resolution using appropriate wavelengths and filters for Cy2, Cy3 and Cy5 dyes. Relative protein quantification across DM1, SHR, SHR/DM1 and control samples was performed using DeCyder software v 7.0 and multivariate statistical module EDA (Extended Data Analysis) (GE Healthcare) in a stepped process. Spots showing significantly altered expression were selected for excision, digestion and analysis by MALDI-MS (Ultraflex MALDI-TOF/TOF mass spectrometer). Mass data were used to search a non-redundant protein database (NCBInr; ~6.5×10^6 entries) using the Mascot software (see supplemental methods). 91 proteins were identified differentially expressed

(Supplementary Table 1). The Student's t test was used to compare average ratios for each spot between DM1, SHR, SHR/DM1 and control samples. P values less than 0.05 were considered significant. BioVenn software was used for protein clustering.

The lists of differentiated proteins, along with their corresponding fold-change ratio and accession number, were uploaded as inputs for IPA® (Ingenuity Systems) and Pathway Architect® (Stratagene, La Jolla, CA, USA) software to identify related biological and molecular networks. IPA and Architect databases are comprised of human, mouse and rat orthologs. Protein networks were algorithmically generated based on their connectivity. A functional classification of the altered proteins was derived from the web-based tool GenCards®. Metabolic [FFA and carbohydrates oxidation, tri-carboxylic acid (TCA)-cycle and mitochondrial respiratory chain], cytoskeleton and apoptosis regulated proteins were categorised and appear in Supplementary Table 1. Miscellaneous proteins were not directly related to the aforementioned processes.

2.3. *Immunohistochemistry (IH)*

Paraffin sections of myocardia (n=6–8, each) were mounted on slides and used for IH. Primary antibodies (anti-LDB3 or anti-ANXA5, Sigma) were incubated overnight at 4 °C, analysed by biotin-labelled secondary antibodies (anti-goat or anti-rabbit, respectively; GE Healthcare) and developed using a streptavidin system (see Supplemental methods). For co-IH assays, both anti-ANXA5 and anti-troponin-T-type2 (Sigma) were added together and developed with their specific secondary antibodies (labelled with Texas-Red-633 and Alexa-Fluor-488 dyes, Sigma). IH staining was semi-quantified by Metamorph software on ten fields of myocardia. Photographs with a bar scale were taken at 40× magnification under microscopy.

2.4. *Western Blot (WB) and Electrophoretic Mobility Shift Assay (EMSA)*

A piece of pulverized ventricle or cell extract was dissolved in protein lysis buffer (see Supplemental Methods). Proteins were separated on polyacrylamide gels, transferred to membranes and probed with specific antibodies. The primary antibodies used were anti-LDB3 (Abcam, Cambridge, UK.), -ANXA5 (Abcam), -HSPB27 (Sta. Cruz Biotechnology, Inc., CA, U.S.A.), -CAT (Calbiochem, Darmstadt, Germany), -HNF4α (Sigma), -PPARα (Sigma) -PPARß/δ (Aviva System Biology, San Diego, CA, U.S.A.) -PDK4 (Sigma) or -PGC1α (Sigma). Anti-GAPDH (Sigma) was unchanged in proteomic analysis in all groups and used as loading control. A representative gel of at least three experiments from all rats and the semi-quantification score (n-fold) are shown.

EMSA and super-shift assays were used to detect the nuclear transcription factor activation (see supplemental methods). Pulverized ventricles or nuclear cell extracts were dissolved in lysis buffer, incubated with radioisotope-labelled (with γ-^{32}P-ATP) oligonucleotides [HNF4α or PPARs (α, ß/δ and γ), Sta. Cruz Biotechnology] and separated on gels. Gels were exposed to X-ray films. Protein (nuclear transcription factor) and oligonucleotide binding were semi-quantified by densitometry. A competition assay for specific binding was carried out by pre-incubating the protein extract (the same as the closest lane) with the unlabeled (see C.) or unrelated (not shown) oligonucleotide, before adding the labeled oligonucleotide. Non-specific probes were similar for all rats or cells (not shown). Super-shift assays were performed to disclose the PPAR isoforms (α or ß/δ) involved in the transcription factor activation. Myocardium or nuclear cell extracts were pre-treated (1 h at 25 °C) with specific anti-PPARα or anti-PPRß/δ antibodies before the γ-[^{32}P]-oligonucleotide incubation and then separated on gels. A super-shifted band indicates the implication of the specific isoform. In the figures we show a representative gel (only over-exposure top part) of at least three experiments from all rats or cells, and the semi-quantification score (n-fold).

2.5. *Cultured cardiomyocytes*

H9c2 were grown in DMEM (with 5 mM D-glucose) supplemented with 10% foetal calf serum (see Supplemental Methods). These cells have been previously used as an *in vitro* model of cardiac diabetes [21]. H9c2 differentiated from mononucleated myoblasts into myocytes upon reduction of serum concentration. We mimicked the hyperglycemic and hypertensive conditions by adding D-glucose (up to the final concentration of 33 mM) or angiotensin-II (10^{-7} M, Sigma) for 24 h. The resulting glucose concentration corresponds to plasma levels of 590 mg/dl. For pre-treatment studies, PPARα-agonist [GW7647 (10 μM), Sta. Cruz Biotechnologies] or PPARα-antagonist [G5045 (5 μM), Sigma] was added 24 h before stimulation. The AngII-AT_1 receptor antagonist (Valsartan, 1 μM; Novartis, Switzerland) or insulin (Humulina NPH, 10nM; Lilly, USA) was added 30 min before stimulation. To evaluate the extent of hypertrophy, cardiomyocyte size was quantified as surface area from 10 randomly chosen fields of actin-stained cells [anti-F-actin phalloidin (1 h, 37 °C); Sigma], in at least three independent experiments. Photographs with a bar scale were taken at 40× magnification under confocal microscopy.

2.6. *Quantitative PCR (QPCR)*

Total RNA was extracted from cultured cardiomyocytes and dissolved in Trizol reagent (Invitrogen). Equal amounts of RNA were reverse-transcripted to obtain the cDNA and used for multiplex QPCR. The QPCR mixture was prepared as follows: 33 ng of cDNA, 0.18 μl of gene expression assays ANP (Rn00561661_m1)-, CPT1b (Rn00682395_m1)-, ACADL (Rn00563121_m1)- or ACADM (Rn00566390_m1)-Fam fluorophores + 0.37 μl housekeeping gene eukaryotic ribosomic 18 s-Vic fluorophore (4310893E)], 5 μl premix buffer (polymerase and salts) and RNAase free water (Applied Biosystems). Amplification conditions were: 2′ at 50 °C, 10″ at 95 °C and 40 cycles of 15″ at 95 °C and 1′ at 60 °C (Applied Biosystems 7500). All samples were prepared in triplicate to obtain their threshold cycle (Ct). Where deviation for each triplicate was higher than 0.3 cycles, Ct was not considered. We show a quantification (–fold ANP vs. 18 s) of three QPCRs from at least three independent assays.

2.7. Statistical analysis

Results are expressed as mean±standard deviation. For proteomic analysis, the Student's t test was used to compare average ratios for each spot between groups ($p<0.05$ was considered significant). For sample clustering, multivariate principal component analysis (PCA) was carried out using an algorithm included in the EDA module of the DeCyder software v7.0 (see Supplemental material). For biochemical approaches, multiple comparisons were performed by a Kruskal–Wallis test followed by a Mann–Whitney test. A two-tailed $p<0.05$ was considered significant.

3. Results

3.1. Cardiac proteome of long-term DM1 and hypertensive rats

After 22 weeks of experimental diabetes, DM1 rats exhibited hyperglycemia, hypoinsulinemia and proteinuria. SHR showed higher systolic blood pressure and similar proteinuria, whereas SHR/DM1 rats showed hyperglycemia, hyperinsulinemia, severe proteinuria and high systolic blood pressure [3]. Then, myocardia were analysed by two-dimension differential in-gel electrophoresis (2D-DIGE). Automated image analysis detected approximately 2650 spots per gel, of which 1848 spots were matched throughout the gels (Fig. S1a). Multivariate PCA showed efficient discrimination between the groups (Fig. S1b) and DeCyder analysis revealed significant changes in the abundance of 24, 53 and 53 proteins in DM1, SHR and SHR/DM1 rats, respectively (ratio≥1.35-fold, $p<0.05$; Fig. S1a). Spots from silver-stained gels (Fig. S1b) were digested for protein identification. The identified molecules and their expression data are summarized in Fig. 1 and detailed in Supplementary Table 1. Some particular proteins were identified in several spots, which can be accounted for by protein post-translational or chemical modifications.

3.2. Differentiated proteins in the myocardium

3.2.1. Metabolism

Metabolic enzymes were the most altered proteins (at least 50%) in DM1 and both hypertensive (SHR and SHR/DM1) hearts.

3.2.1.1. *FFA metabolism*. In DM1 myocardium, ß-oxidation enzymes remained unchanged. However, SHR hearts showed a decrease of mitochondrial tri-functional protein (HADHA), short-chain acyl Co-A dehydrogenase (ACADS) and acyl Co-A synthetase-long chain (ACSL1), -short-chain (ACSS1) and -family member 2 (ACSF2) (Fig. 1). SHR/DM1 rats also had lower expression of ACADS, ACSL1, ACSF2 and, additionally, trans-2-enoyl CoA reductase (MECR). However, L-3-hydroxyacyl-CoA dehydrogenase (HADH) was increased.

3.2.1.2. *Carbohydrate metabolism*. Glycolytic enolase-1α (ENO1) was elevated in DM1, SHR and SHR/DM1 rats. However, downstream in the pathway, pyruvate kinase-2 (PKM2) decreased in both DM1 and SHR hearts, while the component X of pyruvate dehydrogenase (PDHX) augmented only in SHR (Fig. 1). In addition, glycogen phosphorylase (PYGM) was reduced in SHR, whereas UDP-glucose pyrophosphorylase-2 (UGP2) was over-expressed, and L-lactate dehydrogenase B (LDHB) was down-regulated in SHR/DM1 hearts.

Downstream, the mitochondrial TCA-cycle was affected in all rats. Both DM1 and SHR myocardia had a reduction in aconitase-2 (ACO2), isocitrate dehydrogenase-3 (IDH3A), dihydrolipoamide S-succinyltransferase (DLST), succinate CoA-ligase (SUCLG1) and mitochondrial malate dehydrogenase (MDH2). In SHR/DM1, a decrease in DLST and citrate synthase (CS) was observed (Fig. 1). Next, the electron transport chain at the inner mitochondrial membrane was altered. The ATP synthase (ATP5A1) was found to be down-regulated in DM1, SHR and SHR/DM1 hearts. Moreover, ubiquinol–cytochrome C reductase (UQCRC1), a component of the respiratory complex-III, was reduced in DM1 and SHR. In SHR and SHR/DM1, NADH–dehydrogenase ubiquinone 1α-10 (NDUFA10) and cytochrome C1 (CYC1) were also reduced, respectively. However, NADH–dehydrogenase flavoprotein-1 (NDUFV1) was augmented in SHR (Fig. 1). Finally, mitochondrial ATP-carrier creatine kinase (CKMT2) was down-regulated in SHR/DM1 and its cytosolic isoform (CKM) was also decreased in DM1 and SHR. However, a CKM-heterodimer member, creatine kinase-B (CKB), was elevated in both hypertensive hearts.

3.2.2. Cytoskeleton regulation

We previously showed an increase in cardiomyocyte size at this stage of these injuries, with the increase being more prominent in the presence of hypertension [3]. In the current study, proteomic analysis revealed changes in cytoskeletal and contractile proteins (13% of the total altered proteins), mainly in hypertensive hearts. DM1 showed a significant increase in cytoskeletal desmin (DES) and LIM proteins (LDB3 and PDLIM) (Fig. 1). DES, LDB3 and PDLIM, together with another LIM member, cysteine and glycine-rich protein-3 (CSRP3), were also increased in SHR rats. In SHR/DM1, DES, LDB3, CSRP3, and additionally four-and-a-half LIM domains-2 (FHL2), Rho GTPase activating protein-1 (ARHGAP1) and myozenin-2 (MYOZ2) were also augmented. The changes in LDB3 levels were validated by IH and WB (Fig. 2a, c). In addition, the contractile protein cardiac α-actin (ACTC1) was significantly down-regulated in the SHR and SHR/DM1 myocardia. In this sense, myosin light chain-2 (MYL2) was induced in SHR, and myosin heavy chain-6 (MYH6) was elevated in SHR hearts (Fig. 1).

3.2.3. Apoptosis

An increase of pro-apoptotic factors and apoptosis were described in the myocardia of these DM1 and markedly hypertensive rats [3]. Proteomic analyses now show the presence of at least 10% of apoptosis-related factors. In DM1, pro-apoptotic annexin-V (ANXA5) was over-expressed whilst the molecular chaperone heat shock protein 60/65 (HSPD1) was decreased (Fig. 1). SHR showed exacerbated ANXA5 over-expression, and reduction of HSPD1 and a chaperone-inducer named protein disulphide isomerase associated-3 (PDIA3). In SHR/DM1 rats, ANXA5 increased, and PDIA3 and chaperone TNF receptor-associated protein-1 (TRAP1) were down-regulated. However, there was an

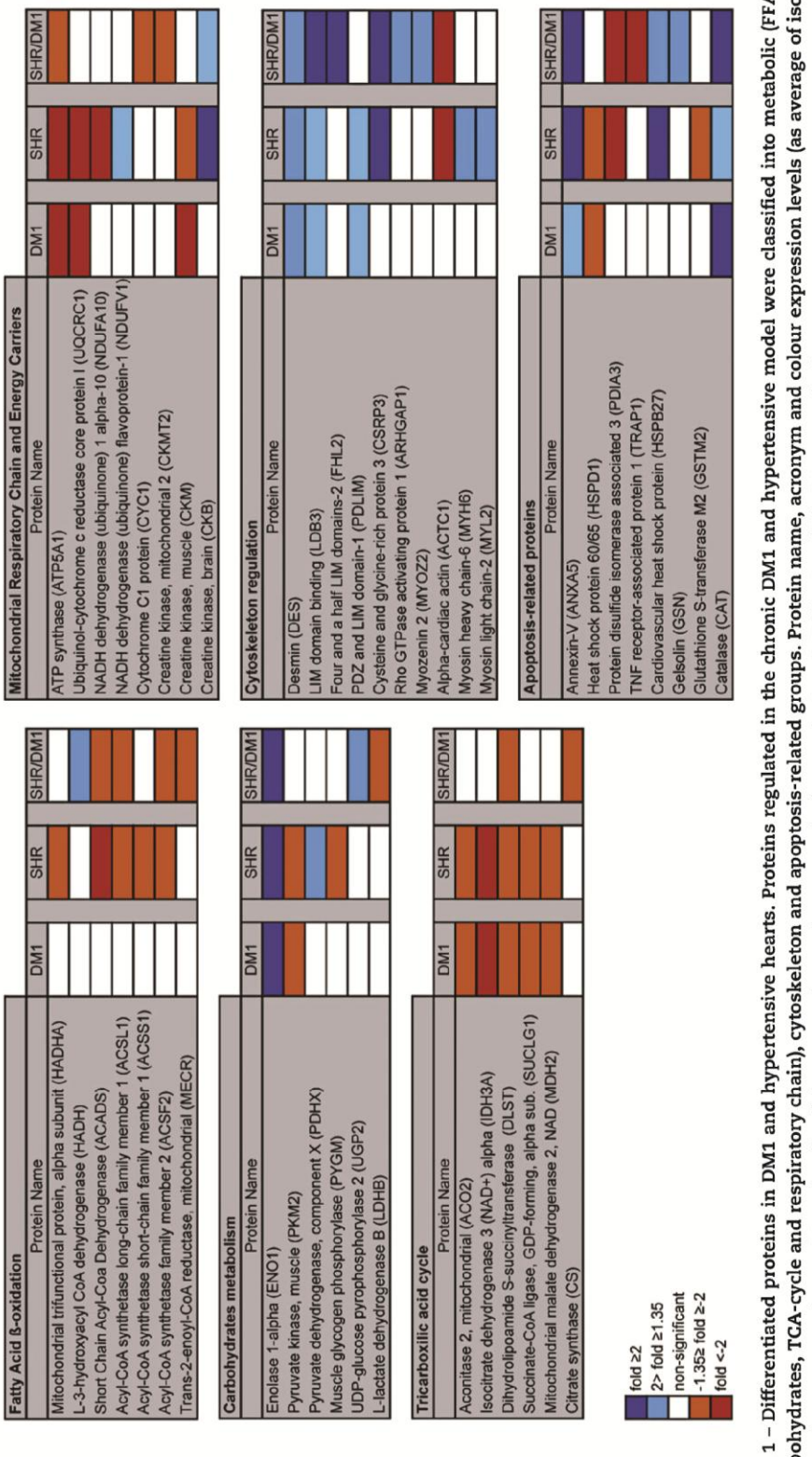

Fig. 1 – Differentiated proteins in DM1 and hypertensive hearts. Proteins regulated in the chronic DM1 and hypertensive model were classified into metabolic (FFAs, carbohydrates, TCA-cycle and respiratory chain), cytoskeleton and apoptosis-related groups. Protein name, acronym and colour expression levels (as average of isoforms) for each group are shown. More details of the expression and spot numbering are in Suppl. Table 1 and Fig.S1, respectively.

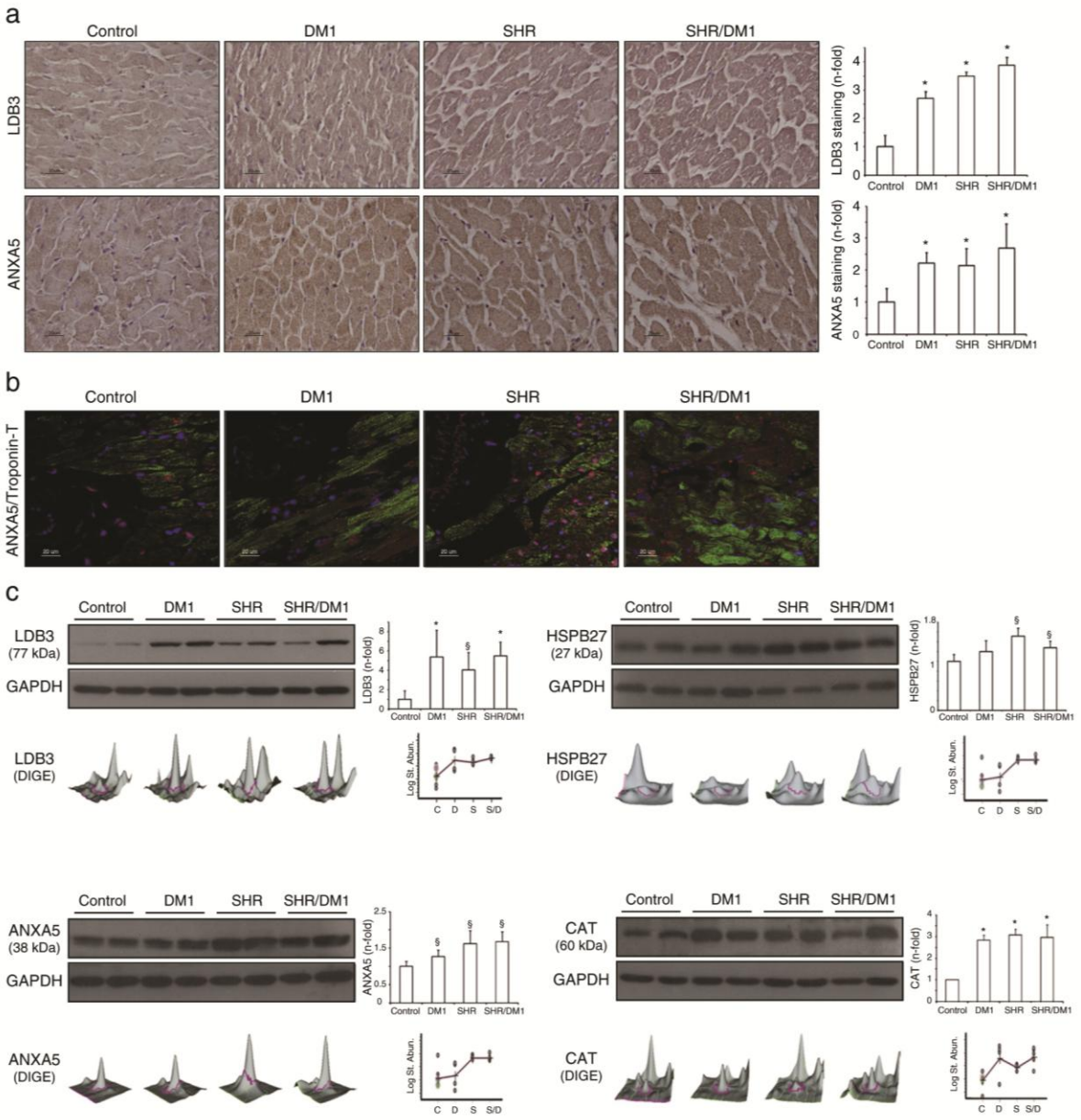

Fig. 2 – Expression levels of LDB3, ANXA5, HSPB27 and CAT. (a) IH analysis of LDB3 and ANXA5 in DM1, SHR and SHR/DM1 hearts. (b) Co-staining of ANXA5 (red) and cardiac troponin-T (green) in the myocardia. DAPI was used for nuclear staining (blue). (c) WB analysis of LDB3, ANXA5, HSPB27 and CAT expression. Semi-quantification graphs are shown. *p<0.01 and §p<0.05 vs. control. In the bottom, DeCyder protein volumes and average standardised log abundance value. C, control; D, DM1; S, SHR and S/D, SHR/DM1.

over-expression of the cardiovascular heat shock protein (HSPB27) observed in SHR, and anti-apoptotic gelsolin (GSN) in SHR/DM1. ANXA5 (Fig. 2a) and HSPB27 (Fig. 2c) changes were confirmed by IH and/or WB, and ANXA5 co-staining with a cardiomyocyte marker (troponin-T) was also shown (Fig. 2b). In addition, anti-oxidant molecules can improve apoptosis. In SHR the detoxification enzyme glutathione S-transferase M2 (GSTM2) was decreased. However, catalase (CAT) was up-regulated in all rats, which was corroborated by WB (Fig. 2c).

3.3. *Predicted pathways activated in DM1 and hypertensive hearts*

The differentiated proteins were analyzed by IPA and Architect software. For the DM1 myocardium (Fig. 3a, left),

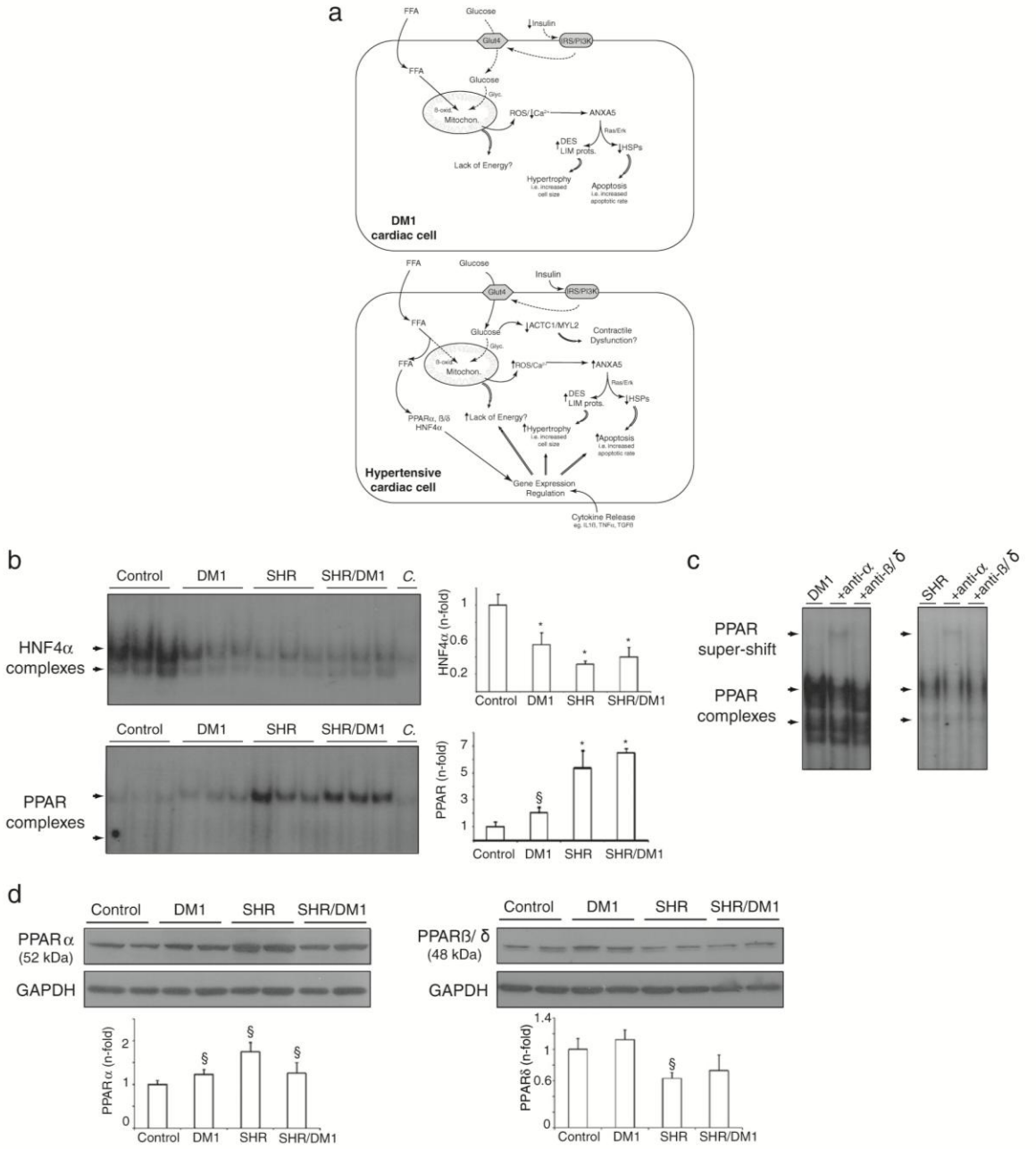

Fig. 3 – Predicted pathways involved in the chronic DM1 (left) and hypertensive (right) myocardia; (a) Different molecular mechanisms may be activated in the DM1 and hypertensive cardiac cells. Dotted arrows represent potential down-regulated pathways. *In vivo* evidence of cardiac hypertrophy, apoptosis and cytokine release were also found in the rats [3]. Regulation of nuclear transcription factors in DM1, SHR and SHR/DM1 hearts; (b) DNA-binding activity for HNF4α and PPARs as revealed by EMSA. Arrows indicate the DNA complexes. *C*: competition assay. Non-specific radioactive probes were similar for all rats (not shown). (c) Over-exposed EMSA for super-shift detection. DM1 and SHR hearts were pre-treated with anti-PPARα or anti-PPARß/δ antibodies. Super-shift bands are arrowed. (d) PPARα and PPARβ/δ levels in WB. Semi-quantification graphs are shown. *$p<0.01$ and §$p<0.05$ vs. control.

bioinformatic studies suggest that the absence of insulin would reduce glucose entrance and mitochondrial metabolism, thus leading to ROS accumulation and a decrease in energy production and Ca^{2+} influx [2,4]. Ca^{2+} disruption and ROS may stimulate ANXA5, which is one of the hypertrophic and apoptotic triggers [22]. Through Ras and Erk

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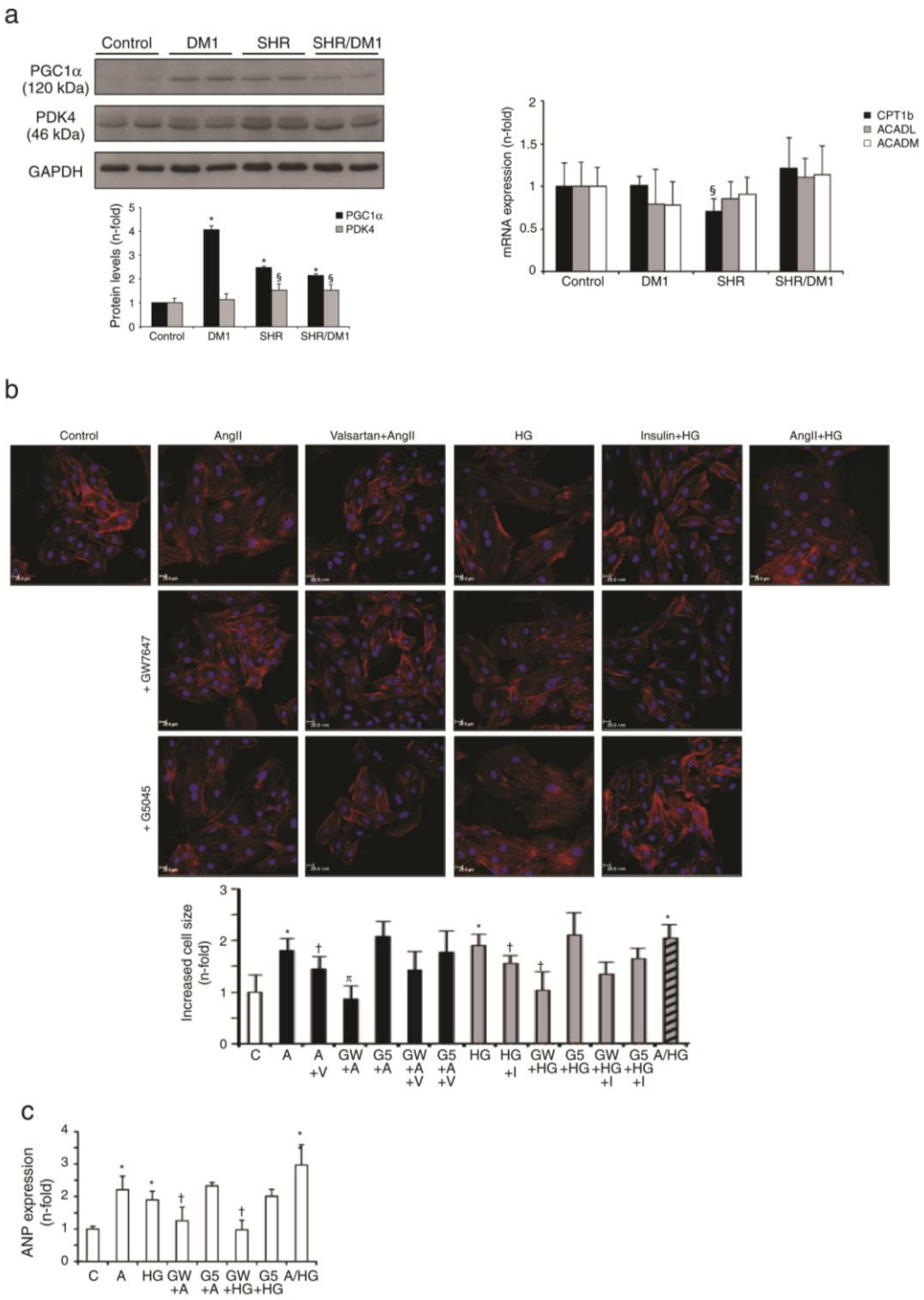

Fig. 4 – PPARα regulation and cardiomyocyte hypertrophy. (a) Expression of PPARα downstream genes: PDK4, CPT1b, ACADL and ACADM in DM1 and hypertensive myocardium. A PPARα co-activator (PGC1α) was also detected in the rats. (b) Immunofluorescence for F-actin (red) in AngII and/or HG stimulated cardiomyocytes. Some cells were pre-treated with an AngII-AT_1 receptor antagonist (valsartan), insulin and/or a PPARα agonist (GW7647) or antagonist (G5045). Nuclei were stained with DAPI (blue). Semi-quantification score of the increased cell size, and (c) expression levels a pro-hypertrophic marker, ANP (by QPCR), is also shown. *p < 0.01 and §p < 0.05 vs. control. πp < 0.01 and †p < 0.05 vs. AngII or HG.

interactions, annexins would lead to the over-expression of DES and LIM-proteins and down-regulation of HSPs [23,24].

Under hypertension (Fig. 3a, right), bioinformatic approaches indicate an impairment of FFA metabolism, which results in their cytosolic accumulation. FFAs could bind to FFAs-nuclear receptors HNF4α (hepatocyte nuclear factor-4α) or PPARs to regulate the expression of specific genes, such as ACADS and ACSL1 (ß-oxidation) [25,26]. Bioinformatic approaches also suggest that this metabolic alteration, together with the glycolysis defect may trigger ROS generation (and Ca^{2+} disruption), which stimulates ANXA5 for hypertrophic and apoptotic responses. Also, elevated glucose metabolites would induce the isoform switch between ACTC1 and myosins such as MYL2, affecting cell contractility [27]. A release of specific cytokines to exacerbate these responses also appears as a predictive event in this heart. Interleukin IL1ß, via Ca2+ signals, may down-regulate glycolysis [28] and respiration [29] enzymes. IL1ß could also induce pro-apoptotic TNFα, which leads to over-expression of myosins and pro-hypertrophic/fibrotic TGFß1 [30,31]. Finally, like DM1 heart, survival factors such as HSPB27 and CAT may be also induced [32,33].

3.4. *Cardiac activation of FFA-receptors in DM1 and hypertension*

Since bioinformatic analysis suggested a key function for transcription factors HNF4α and PPARs in the context of DM1, SHR and SHR/DM1 pathologies, their activation and potential function were investigated. EMSA revealed reduced HNF4α and increased PPARs DNA-binding in the DM1 and pronouncedly, SHR and SHR/DM1 myocardia (Fig. 3b). In particular, super-shift assays showed a shifted b and when DM1 and SHR heart extracts were pre-treated with the anti-PPARα antibody, but not with anti-PPARß/δ (Fig. 3c). As expected, only PPARα was over-expressed in the rats (Fig. 3d). Thus, we tested whether PPARα were truly active in the hearts by analysing the expression of recognised PPARα-regulated genes [25–27]. According to PPAR-DNA binding and expression data, pyruvate dehydrogenase kinase-4 (PDK4) was over-expressed principally in SHR and SHR/DM1 myocardia (Fig. 4a, left). Also, a transcriptional PPARα co-activator, PGC1α (PPARγ co-activator-1α), was increased in these hearts. However, the expression of PPARα-regulated genes of FFA-oxidation CPT1b (carnitine pamitoyltransferase-1), ACADL and ACADM (long and medium chain acyl-coenzyme A dehydrogenase) was not up-regulated (Fig. 4a, right), according to the data achieved by proteomics. In this regard, neither HG nor AngII induced an elevation of CPT1b, ACADL and ACADM in cultured cardiomyocytes (not shown).

3.5. *Fatty-acid receptors and hypertrophy under hyperglycemic and hypertensive conditions*

PPARα activation has been linked to the attenuation of cardiac hypertrophy [17–19]. Thus, we investigated whether PPARα

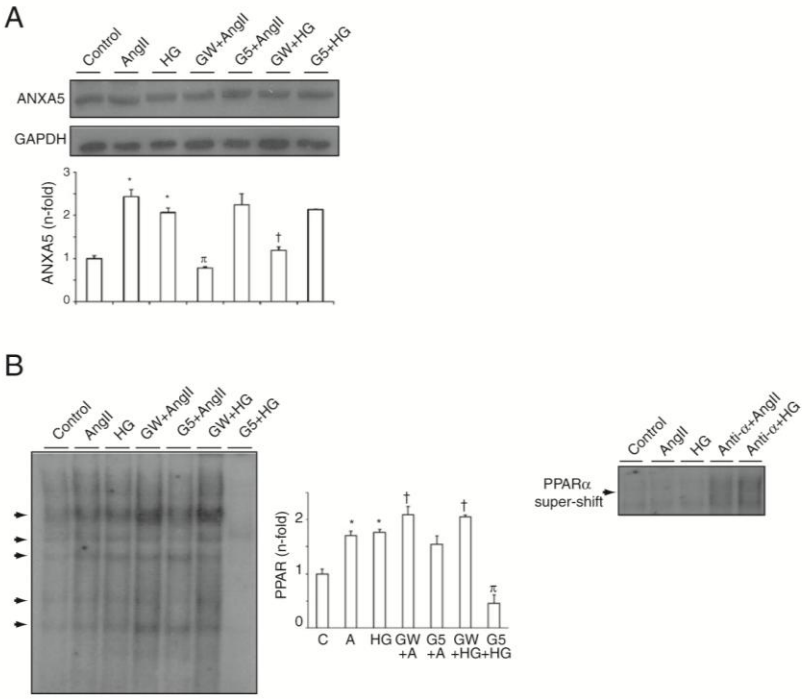

Fig. 5 – Pro-hypertrophic expression and PPARα activation in cardiomyocyte stimulated cells. (a) Expression of ANXA5 (by WB) in AngII or HG incubated cardiomyocytes. Some cells were pre-treated with a PPARα agonist (GW7647) or antagonist (G5045). (b) PPAR DNA-binding revealed by EMSA, and PPARα detection by super-shift assay (only the upper gel area) in AngII or HG stimulated cardiomyocytes. DNA-complexes and super-shift bands are arrowed. *p<0.01 and §p<0.05 vs. control. πp<0.01 and †p<0.05 vs. AngII or HG.

could be involved in the hypertrophic response to cardiac diabetes and hypertension. In cardiomyocytes, HG and AngII induced cell hypertrophy (Fig. 4b) and pro-hypertrophic factor ANP (atrial natriuretic peptide) expression (Fig. 4c). The HG or AngII-induced hypertrophy was prevented by insulin or valsartan (an AngII-AT_1 receptor antagonist) treatments, and no additional effects were observed when both stimuli were added together. Interestingly, ANXA5, an important factor involved in cardiac hypertrophy [24], was also increased after HG and AngII incubation (Fig. 5a). These data are in concordance with the increase of hypertrophy [3] and ANXA5 expression (Figs. 1 and 2) observed in DM1 and hypertensive hearts. Moreover, HG and AngII incubation induced PPAR DNA-binding (Fig. 5b), particularly PPARα isoform (Fig. 5b, right), and the pre-treatment with a PPARα agonist (GW7647) increased PPAR DNA-binding (Fig. 5b) and reduced both cell hypertrophy (Fig. 4b) and pro-hypertrophic molecules ANP and ANXA5 (Figs. 4c and 5a).

4. Discussion

In chronic DM1 we have found 24 proteins to be significantly changed, whereas roughly twice this number was seen in both SHR and SHR/DM1 hearts. These differentiated proteins may be linked to specific biological responses.

4.1. *Metabolic changes in DM1 and hypertensive hearts*

Glucose is the preferred cardiac substrate under pathological conditions. However, in type-I diabetes, glucose assimilation is impaired. In early DM1, proteomic studies disclosed ENO1 over-expression alongside a decrease in downstream PDH decrease [7,8]. In long-term DM1 we showed up-regulation of ENO1, but also inhibition of PKM2 (Fig. 6a). In hypertensive rats, we confirmed an increase of ENO1 [12,13], but additionally a PDHX elevation and PKM2 inhibition. This suggests an uncoupling of glycolysis and pyruvate oxidation resulting in an accumulation of glycolytic intermediates (Fig. 6b). These metabolites may deviate to AGEs (advanced glycation end-products), ROS and ribose/hexosamine pathways, which regulate glucose sensing genes. Indeed, PKM2 and ACTC1 expression is down-regulated by glucose intermediates [27,34]. Thus, DM1 and, especially, hypertensive hearts may be exposed to deleterious substances and develop contractile dysfunction. In this sense, we have demonstrated a switch of contractile proteins to a foetal-like pattern (increased MYL2/ACTC1 ratio) and up-regulation of ANP (not shown) in SHR rats. Studies in rodent models of cardiac injury have led to the description of this behaviour as an adaptive response to maintain contractile function [27].

FFAs will remain the major energy supplier in type-I diabetes. In early DM1 hearts, some authors observed either over-expression [9,10] or down-regulation [11] of ß-oxidation enzymes. In long-term DM1 we did not observe significant changes in these proteins. Indeed, CPT1b, ACADL and ACADM were also not altered in the DM1 hearts or in HG-induced cardiomyocytes. DM1 hearts, at this stage of the disease, may keep normal levels of ß-oxidation to degrade FFA as the only source of ATP, doing so without producing additional ROS (Fig. 6a). Martens et al. also demonstrated non-changes in ß-oxidation enzymes of pancreatic cells exposed to hyperglycemia [35]. However, ß-oxidation could be reduced in chronic hypertension. HADHA and carnitine pamitoyltransferase-2, among others, were shown to be either up-regulated (in early injury) or down-regulated (in late injury) [14]. Our results confirm a decrease of HADHA but also ACADS, ACSF2 (and CPT1b). ACSL1 and MECR, two more newly identified proteins by proteomics, were also reduced (Fig. 6b). Thus, ß-oxidation amelioration could lead to the deviation of FFAs to toxic products and/or transcription factor binding (see below).

Downstream, the TCA-cycle, respiratory chain and ATP-carriers could be attenuated due to an ineffective glycolysis and/or ß-oxidation. We confirmed previous data [7–14] indicating down-regulation of most TCA and respiratory enzymes in both DM1 and hypertension. Moreover, bioinformatics analyses suggest that specific cytokines could intensify the TCA/respiratory reduction in the hypertensive heart [36]. We previously showed IL1ß, TNFα and TGFß elevation in these SHR samples [3]. Thus, hypertensive hearts may accentuate the energy-starvation.

4.2. *Cardiac hypertrophy and apoptosis in DM1 and hypertension*

Early experimental DM1 and both early and late hypertensive hearts exhibited cell hypertrophy and apoptosis [7–14]. We observed the same responses in long-term DM1, SHR and SHR/DM1 hearts [3], adding new data thanks to proteomics. Cardiac hypertrophy can be considered a maladaptive response since the increase of cytoskeleton proteins causes cell stiffness and death [2,24]. In this sense, we showed that DM1 and mostly hypertensive hearts presented ANXA5 elevation. ANXA5, a new proteomic-detected molecule, is also associated with intercalated discs acting as regulators of Ca^{2+}-handling proteins such as chaperones [24]. These chaperones can render resistance to apoptosis through protein re-folding and oxidative-stress equilibrium [37]. We showed that DM1 and mainly hypertensive hearts presented a reduction of specific HSPs, together with other anti-apoptotic proteins (PDIA3, TRAP1). Thus, cardiac apoptosis may be mediated at least in part by the elevated ANXA5/anti-apoptotic ratio in these pathologies. In this regard, ANXA5 up-regulation was associated with left ventricular hypertrophy and systolic dysfunction in hypertensive patients [38]. In addition, the lack of energy, glucose/lipid intermediates and ROS can induce hypertrophy and apoptosis through ANXA5 expression [2,37]. *In vitro*, we also observed an increase of cell size and ANXA5 (and ANP) expression in HG and AngII-induced cardiomyocytes. Other pro-hypertrophic factors like DES and LIM-proteins were also over-expressed in DM1 and hypertensive hearts. LDB3, a molecule newly detected by proteomics, could be stimulated by lipid accumulation (as occurs in DM1 and hypertension) [4,39] and function as a scaffold to assemble interacting proteins (i.e. ACTC1, MYOZ) during cell hypertrophy [40].

4.3. *Role of PPARα in the chronic diabetic cardiomyopathy*

The accumulated FFA in the DM1 and mainly hypertensive hearts may play compensatory responses by binding to

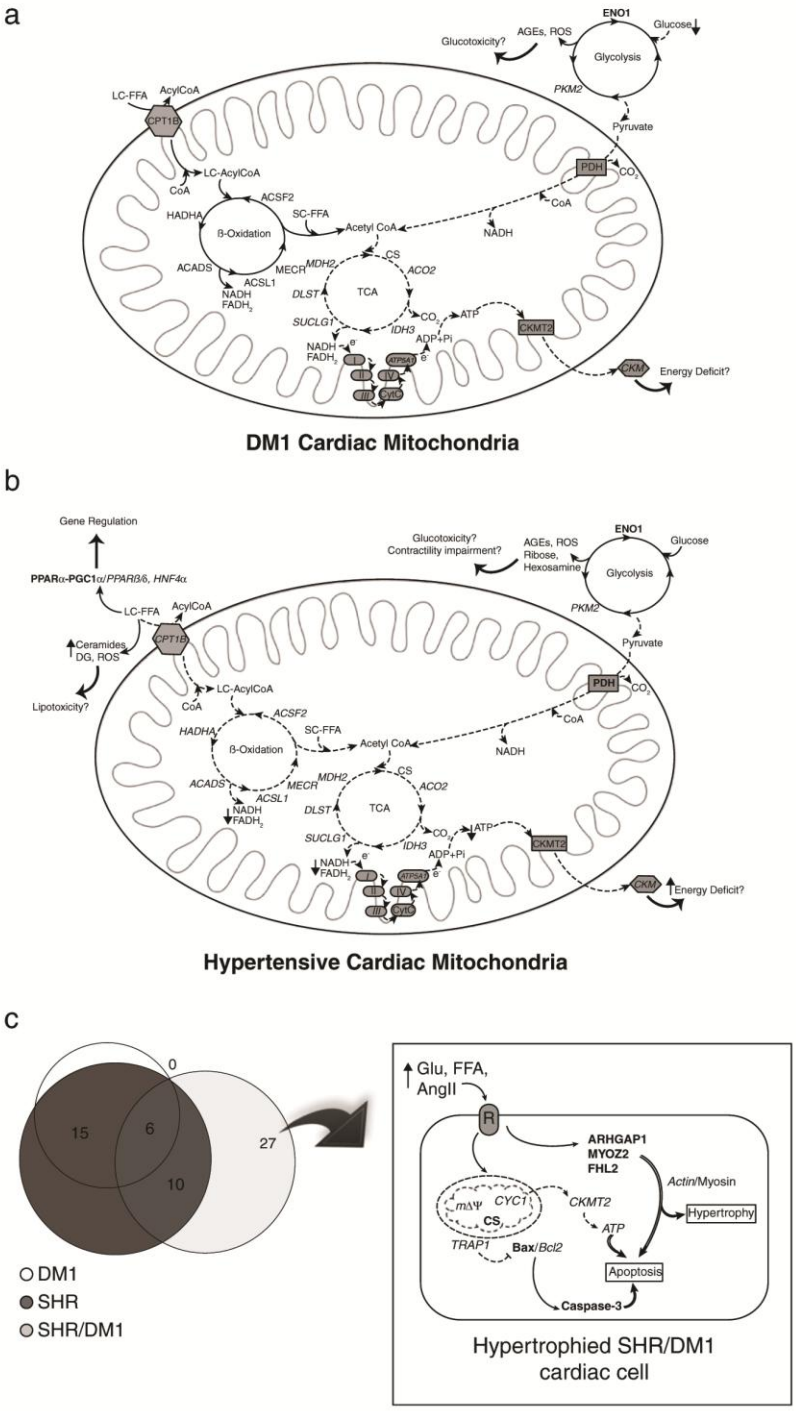

Fig. 6 – Mitochondrial alterations in DM1 and hypertensive hearts. FFA and glucose metabolism in chronic DM1 (a) and hypertensive (b) cardiac cells. Dotted arrows represent down-regulation of the pathways. Bold and italics denote up- and down-regulated levels, respectively. DG, diacylglycerol; LC-FFA, long-chain FFAs; SC-FFA, short-chain FFAs. (c) Distribution of altered proteins in DM1 and hypertensive hearts. Venn diagram showing the total number of "unique" differentiated proteins. DM1, SHR and SHR/DM1 presented 23, 46 and 43 changed proteins, respectively. Fifteen proteins were simultaneously regulated in DM1 and SHR, ten in SHR and SHR/DM1, and six in the three groups. None were only regulated simultaneously in DM1 and SHR/DM1 hearts, and twenty-seven were exclusively found in SHR/DM1. On the right, potential biochemical processes specifically activated in the SHR/DM1 phenotype. mΔΨ, for mitochondrial membrane potential and R, for specific receptors. Bold and italics represent elevated or decreased levels, respectively.

PPARα receptors. We have described up-regulation and DNA-binding increase of PPARα together with over-expression of a PPARα co-activator (PGC1α) and a PPARα-downstream gene (PDK4), mostly in hypertensive myocardia. These data suggest activation of PPARα, which confirms previous data in old SHR rats [41]. However, PPARα may not be focused to stimulate ß-oxidation since CPT1b, ACADL and ACADM were also not elevated in hypertensive hearts. Thus, as hypothesized earlier [41], at these stages of DM1 and hypertension, PPARα may lead to other responses such as repression of hypertrophy in the myocardium. We have described an attenuation of HG- and AngII-induced pro-hypertrophic expression (ANP and ANXA5) and increased cell size, by a PPARα-agonist treatment in cardiomyocytes. Similarly, several PPARα-agonist treatments exerted protective anti-hypertrophic properties by direct effect on cardiomyocytes [42,43] and indirectly via monocyte signalling and increased endothelial NO production [44]. Also, stimulation of PPARα improved fibrosis and cardiac dysfunction in diabetic [19] and hypertensive [44] rats. Altogether, PPARα stimulation could control the expression of metabolic, fibrotic and also hypertrophic genes as adaptive mechanisms of cardio-protection against DM1 and hypertension. In this sense, the over-expression of PGC1α in DM1 and hypertensive hearts may also response to stimulate mitochondrial biogenesis and glucose uptake (by interaction with MEF2 transcription factor) [15].

Nevertheless, PPARα may antagonize hypertrophy and stimulate ß-oxidation at different times, or that specific co-inhibitors (or lack of co-activators) could interfere with PPARα activity on ß-oxidation by trans-repression mechanisms [45,26]. In this regard, PPARß/δ and HNF4α were repressed in DM1 and principally, in hypertensive hearts. A counterbalance between PPARα and PPARß/δ was also seen in hypertensive rat vessels and type-II diabetic hearts [26,46]. Nagatomo et al. observed an increase of PPARα, decrease in PPARß/δ, and no changes in CPT1b and ACADM levels from SHR [41]. Also, HNF4α reduction has been described in liver and kidney of DM1 and hypertensive rats [26]. HNF4α is a master transcription factor involved in the control of fat and carbohydrate metabolism. While the role of HNF4α in hypertension is unknown, HNF4α mutations lead to "maturity-onset diabetes of the young" [47]. Also, the increase of PGC1α may facilitate PPARα interactions with other transcription factors such as SRC-1 or p300, and then, control of hypertrophy [15]. Thus, the alteration of PPARß/δ, HNF4α and/or PGC1α levels could preclude PPARα from ß-oxidation activation or may trigger PPARα for pro-hypertrophy repression. Further experiments are needed to elucidate the relationship between PPARα and its interacting partners in the activation of specific responses within the progression of diabetic cardiomyopathy.

4.4. *Co-existence of chronic DM1 and hypertension in the heart*

In this model, SHR and SHR/DM1 myocardia showed similar modulation of fibrosis and inflammation events. However, cardiomyocyte size was higher in SHR/DM1 (208.7% vs. Wistar) than SHR (170.3%) or DM1 (124.3%) [3]. Also apoptosis and expression of pro-apoptotic molecules (FasL, Bax/Bcl2, caspase-3) were higher in SHR/DM1. In this sense, none of the altered proteins in SHR/DM1 were shared by DM1 and 27 non-shared "unique" proteins were revealed only in SHR/DM1 hearts (Fig. 6c). ARHGAP1, MYOZ2 and FHL2, three cell growth-inducer factors, were specifically over-expressed in SHR/DM1 and may enhance the hypertrophic event. FHL2 can also be an apoptosis trigger since it lacks the activity of sphingosine kinase-1 (SK1) in the myocardium [48]. Furthermore, SHR/DM1 heart showed a robust decrease of TRAP1, a key mitochondrial chaperone for the heart [49]. After pressure overload and hypoxia the overexpression of TRAP1 prevented cardiac hypertrophy, depolarization of the mitochondrial membrane and apoptosis [49,50]. In addition, we noted that only SHR/DM1 hearts reduced CYC1 and CKMT2, which transfer ATP from mitochondria to cytosol. Thus, the combined presence of both SHR and DM1 phenotypes may lead to additional remodelling and lethal effects on the cardiac cytoskeleton and mitochondria (Fig. 6c). In this regard, CS, a TCA enzyme widely used as an indirect estimation of mitochondrial mass and toxicity, was specifically reduced in SHR/DM1 rats. Thus, DM1 could bring about a negative influence on the hypertrophic and apoptotic response to the hypertensive-injured heart. Further studies are needed to disclose the role of the different proteins detected only in SHR/DM1 hearts.

5. Conclusions

Using proteomics, chronic DM1 and markedly hypertensive hearts showed altered expression of metabolic, hypertrophic and apoptotic proteins, which were not previously described in early injury. In DM1, glycolytic enzymes were reduced whereas pro-hypertrophic factors such as ANP and ANXA5 were activated. However, both SHR and SHR/DM1 exhibited an additional decrease of ß-oxidation enzymes. In particular, SHR/DM1 myocardium showed enhancement of proteome changes, especially in the stimulation of hypertrophy and apoptosis. Specific alterations detected only in SHR/DM1 hearts may require further investigations. In addition, at these stages of the diseases, PPARα could be activated for different responses to FFA degradation. In this sense, a PPARα ligand attenuated the HG- and AngII-induced cell hypertrophy, ANP and ANXA5 up-regulation in cardiomyocytes. Thus, under chronic hyperglycemia and mainly hypertension, the myocardium could induce PPARα activation, thereby attenuating the induced hypertrophy. However, this process may be orchestrated by specific interacting PPARα partners such as PGC1α, HNF4α or PPARß/δ. Exogenous administration of PPARα agonists may serve as anti-hypertrophic therapy in these pathologies.

6. Study limitations

An echocardiographic examination would have provided a functional correlation of the proteomic alterations found. Previous data in rats showed both systolic and diastolic dysfunctions after 8 weeks of DM1 [7], or hypertension [14]. Thus, in our model analysed after 22 weeks of injury, cardiac dysfunction could be present. Also, *in vitro* regulation of PPARα by specific ligands cannot be directly extrapolated to

an *in vivo* situation. Although this approach has been extensively assayed [16–19,42,43,46] non-myocyte cells may interfere the PPAR signal in the heart. Finally, low-abundance proteins with potentially relevant functions may have been overlooked by proteomic approaches.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2011.12.023.

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ONLINE SUPPLEMENTAL MATERIALS

Supplemental Methods:

Differential in-gel electrophoresis (DIGE)

Two-Dimension (2D)-DIGE sample preparation

A piece of frozen left ventricles from DM1 normotensive, SHR, SHR/DM1 and control rats (n=6) were nitrogen-cold powdered, dissolved in standard cell lysis Buffer. These samples were obtained from the same animals than previous publication [1]. After centrifugation 5 min to 12,000 rpm to remove solid material, supernatants were collected and interfering components were removed using the 2D Clean-Up Kit (GE Healthcare). Protein pellets were solubilized in standard cell Lysis Buffer, and protein concentration was measured with the RC-DC Protein Assay Kit (Bio-Rad).

2D-DIGE separation

Proteins were labeled according to the CyDye manufacturer (GE Healthcare). Briefly, 50 µg of myocardial DM1, SHR, SHR/DM1 or control protein extracts were minimally labeled with 400 pmol of the *N*-hydroxysuccinimide esters of Cy3 or Cy5 fluorescent cyanine dyes on ice in the dark for 30 min. All experiments comprised an internal standard containing equal amounts of each cell lysate, which was labeled with Cy2 dye. The labeling reaction was quenched with 1 µL of 10 mM lysine on ice for 10 min in the dark. A pair of DM1, SHR, SHR/DM1 and control protein extracts, and the internal standard protein samples, were combined and run in a single gel (150 µg total proteins). Protein extracts were diluted in Rehydration Buffer (7 M urea, 2 M thiourea, 4% CHAPS, bromophenol blue traces) containing 50 mM DTT and 0.8% IPG3-11NL Buffer up to final volume of 80 µl, and applied by cup-loading to 24 cm IPG strips pH 3-11 (GE Healthcare) previously rehydrated with 400 µl of Rehydration Buffer containing 100 mM hydroxyethyldisulphide (HED, DeStreak solution; GE Healthacre) and 0.8% IPG3-11NL

Buffer. The first dimension was run at 0.05 mA/IPG strip in the IPGphor IEF System (GE Healthcare) following a voltage increase in 4 steps: 300 V for 3 h, linear gradient to 1000 V in 4 h, linear gradient to 8000 V in 2 h and 8000 V until steady state (around 40,000 Vh). After the first dimension, strips were equilibrated in the dark with SDS Equilibration Buffer [(75 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of bromophenol blue)] containing 1% (w/v) DTT for 15 min and thereafter in SDS Equilibration Buffer containing 4% (w/v) iodoacetamide for 15 additional min. Then the proteins were separated on home-casted 10% Tris–glycine gels using an Ettan Dalt Six device (GE Healthcare) at 25ºC until the tracking dye had migrated off the bottom of the gel.

Image acquisition and analysis

After electrophoresis, the gels were scanned and relative protein quantification across DM1, SHR, SHR/DM1 and control samples was performed using DeCyder software and multivariate statistical module EDA (GE Healthcare) in a stepped process. First, a Differential In-gel Analysis (DIA) module was used to co-detect the 3 images of a gel (internal standard and two samples) to measure accurate spot ratios of the Cy3 and Cy5 spot volumes referring to the standard Cy2 volume on each gel. Background subtraction, quantification and normalization were automatically applied with low experimental variation (DeCyder Differential Analysis Software User Manual, version 7; GE Healthcare, 2009). Then those images individually processed with the DIA module were matched between gels with the Biological Variation Analysis (BVA) module, using the internal standard for gel-to-gel matching. BVA revealed the differences between experimental groups across all the gels. Each difference was calculated as average ratios for each spot. The Student's t test was used to compare average ratios for each spot between DM1, SHR, SHR/DM1 and control samples. P values less than 0.05 were considered significant. Multivariate analysis for sample clustering was performed by PCA (Principal Component Analysis) using an algorithm included in the EDA module of the

DeCyder software v 7.0. PCA reduces the dimensionality of 2D-DIGE multidimensional analyses, displaying the two principle components that distinguish between the two largest sources of variation within the dataset. After imaging for CyDye components and DeCyder analysis, the gels were stained with a compatible silver staining method for protein visualization and identification (Supplementary Table 1).

In-gel trypsin digestion

The same gels scanned for DIGE analysis were later staining with an MS-compatible silver staining protocol [2]. Protein spots were excised from the gels manually and transferred to pierced V-bottom 96-well polypropylene microplates (Bruker Daltonik, Bremen, Germany) loaded with ultrapure water. The samples were digested automatically using a Proteineer DP robot (Bruker Daltonik) under the control of dpControl 1.2 software (Bruker Daltonik) [3] with minor variations: gel plugs were submitted to reduction with 10 mM dithiothreitol (GE Healthcare) in 50 mM ammonium bicarbonate (99.5% purity; Sigma, St. Louis, MO, USA) and alkylation with 55 mM iodoacetamide (Sigma) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 8 ng/ml in 50 mM ammonium bicarbonate was added to the dried gel pieces and the digestion proceeded at 37 ºC for 8 h. Finally, 0.5% tri-fluoroacetic acid (99.5% purity; Sigma) was added for peptide extraction, and the resulting digestion solutions transferred by centrifugation to V-bottom 96-well polypropylene microplates (Greiner Bio-One, Frickenhausen, Germany).

Mass Spectrometry

MALDI samples were prepared by mixing equal volumes of the above digestion solution and a matrix solution composed of a-cyano-4-hydroxycinnamic acid (Bruker

Daltonik) in 50% aqueous acetonitrile and 0.25% tri-fluoroacetic acid. This mixture was deposited onto a 600 mm AnchorChip pre-structured MALDI probe (Bruker Daltonik) and allowed to dry at room temperature [3]. Samples were automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) with an automated analysis loop using internal mass calibration, under the control of flexControl 2.2 software (Bruker Daltonik). In a first step, the MALDI-MS spectra were acquired by averaging 300 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 4000 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with m/z=842.510 and m/z=2211.105. In a second step, precursor ions exceeding a threshold signal-to-noise ratio in the MALDI-MS mass spectrum were subject to fragment ion analysis in the tandem (MS/MS) mode. Precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector to average 1000 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flexAnalysis 2.2 software (Bruker Daltonik). MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or re-labeled when necessary using the above programs as well as home-made software.

Database searching

MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a non-redundant protein database (NCBInr; ~6.5 x 10^6 entries; National Center for Biotechnology Information, Bethesda, US; or SwissProt; ~3.7 x 10^5 entries; Swiss Institute for Bioinformatics, Switzerland) using the Mascot software (Matrix Science, London, UK). Other relevant search parameters were set as follows:

enzyme, trypsin; fixed modifications, carbamidomethyl; allow up to one missed cleavages; peptide tolerance ±20 ppm; MS/MS tolerance ±0.5 Da.

Western Blot (WB)

For WB, a piece of cold-pulverized ventricle or cell extract were dissolved in lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 2% SDS + 1/250 protease inhibitors) and vortex for 30 min on ice. Supernatant (total protein extract) was separated and quantified by the BCA method (Pierce). Thirty μg of samples was loaded on an acrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk in TBST (TBS + tween-20 0.05%) and incubated (overnight at 4°C) with the primary antibody. Next, membranes were incubated with HRP-labeled secondary antibody (GE Healthcare) and developed with ECL/x-ray films. A representative gel and the semi-quantification score are shown.

Electrophoretic Mobility Shift Assay (EMSA)

A frozen pulverized ventricle or nuclear cell extract were, dissolved in lysis buffer (20mM Hepes pH 7.5, 20% Glycerol, 0.35M NaCl, 5mM $MgCl_2$, 0.1 mM EDTA, 1/250 cocktail protease inhibitors) and vortex for 30 min on ice. HNF4α and PPARs (α, ß/δ and γ) were labeled with γ-[^{32}P]-ATP, incubated 30 min with 10 μg cellular extracts in binding buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) and separated on acrylamide gels. Gels were run, dried out and exposed to x-ray films. Protein and oligonucleotide DNA-binding was se mi-quantified by densitometry.

Immuno-histochemistry (IH)

Myocardium from p-formaldehyde samples was embedded in paraffin. Four μm paraffin sections were fixed on slides and used for IH. Tissue internal peroxidase activity and unspecific sites were blocked with 3% methanol-H_2O_2 and blocking buffer (5%

albumin and 10% specific serum in TBS), respectively. Primary antibody was added to the sections in blocking buffer and incubated overnight at 4°C. After washing, secondary biotin-labeled antibodies were added, washed and developed with AB streptavidin-complex and DAB chromogen.

Cardiomyocyte culture

H9c2(2-1) is a permanent myoblast cell line derived from embryonic BD1X rat heart tissue (ATCC; VA, USA). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (BioWhittaker; Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (BioWhittaker), 100 IE/ml sodium penicillin (Yamanouchi Europe; Leiderdorp, The Netherlands), 100 µg/ml streptomycin (Radiopharma-Fisiopharma), 2 mM L-glutamine (GIBCO-BRL; Paisley, UK) and 5 mM D-glucose (Sigma). Properties of H9c2 cells are similar to those in adult cardiomyocytes [4]. Near confluence ($0.9x10^5$ cells/cm^2), H9c2 were differentiated to myocytes upon reduction of serum concentration. During this differentiation process, cells retain several elements of the electrical and hormonal signaling pathway of cardiac cells and have therefore become an accepted *in vitro* model to study the effects of diabetes on the heart [5].

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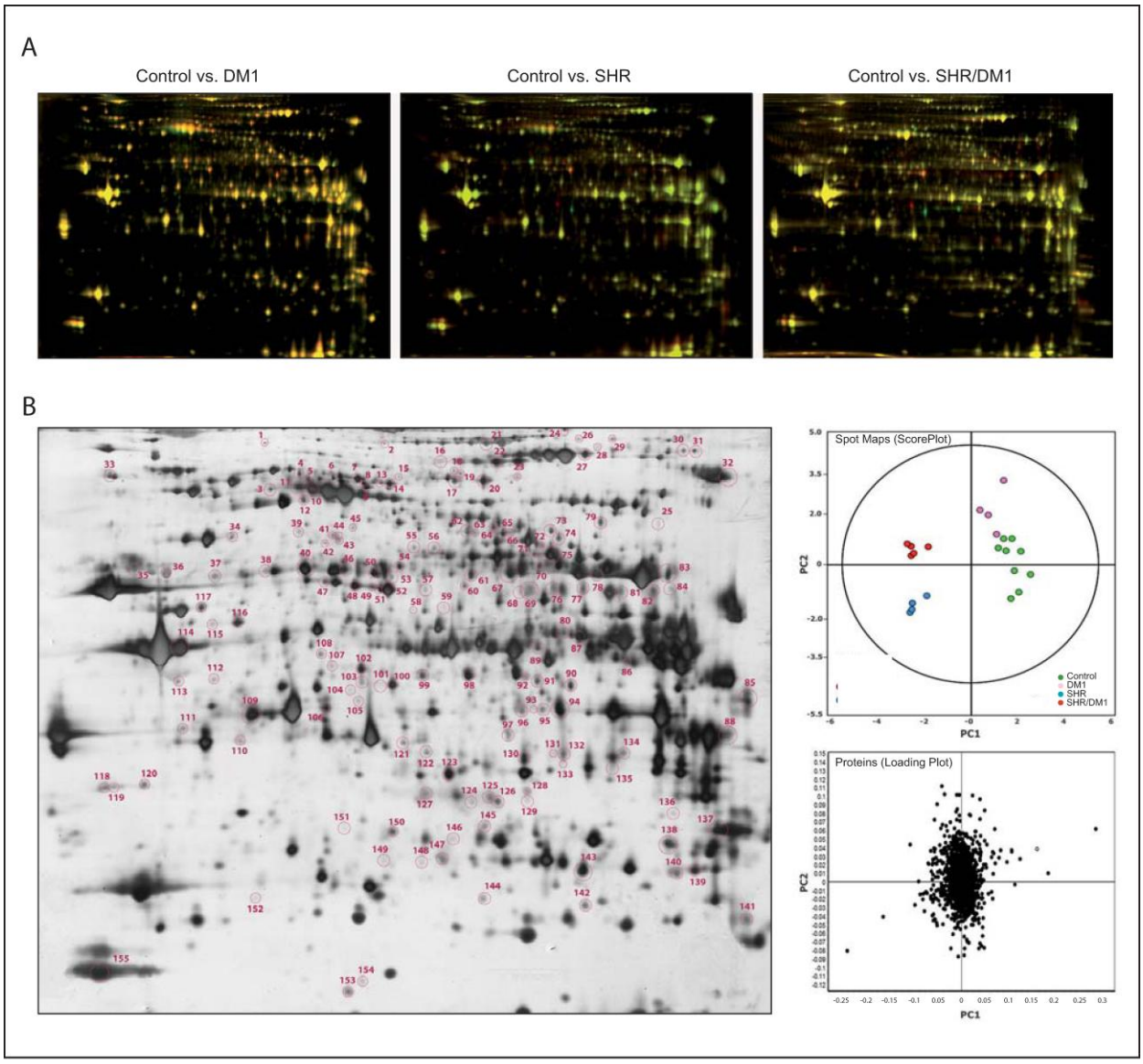


Figure S1.

Figure S1. 2D-DIGE of DM1, SHR, SHR/DM1 and control rats. (a) Myocardial protein extracts from DM1, SHR, SHR/DM1 and control samples and a pooled internal standard were labelled with CyDye. Samples were mixed and separated on 2D gels using IPG strips pH3-11NL (first dimension; left to right), and 10% SDS-PAGE gels (second dimension). The image channels are shown in red (Cy5), green (Cy3) and yellow (overlay). **(b**) Left, a representative silver-stained gel from one of the DIGE analyses used for protein identification showing the identified spots. Right, a PCA considering the spots present across all DIGE gels evidences proper clustering of individual samples by condition.

Fatty acid ß-oxidation

Spot number[a]	Accession code[b]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		SHR/DM1 vs. C[k,l]	
32	gi|148747393	Mitochondrial trifunctional protein, alpha subunit	HADHA	173	3,90E-11	83297	9,16	8	10	-	-	**-1,54**	2,81E-03	-	-
132	gi|1125026	L-3-hydroxyacyl-Coenzyme A dehydrogenase	HADA	135	2,30E-07	34540	8,83	3	8	-	-	-	-	**1,65**	0,0016
98	gi|11968090	Acyl-Coenzyme A dehydrogenase, short chain	ACADS	730	7,20E-67	45224	8,47	12	28	-	-	**-3,36**	0,00412	**-1,37**	3,08E-03
99	gi|11968090	Acyl-Coenzyme A dehydrogenase, short chain	ACADS	197	1,50E-13	45224	8,47	6	16	-	-	**-2,26**	6,26E-03	**-1,96**	6,66E-04
17	gi|25742739	Acyl-CoA synthetase long-chain family member 1	ACSL1	585	2,30E-52	79155	6,6	24	43	-	-	**-1,52**	1,20E-03	**-1,37**	7,22E-03
23	gi|25742739	Acyl-CoA synthetase long-chain family member 1	ACSL1	165	2,50E-10	79155	6,6	8	12	-	-	**-1,69**	0,017	-	-
14	gi|157818027	Acyl-CoA synthetase short-chain family member 1	ACSS1	395	2,50E-33	75528	6,38	9	13	-	-	**-1,51**	0,0219	-	-
64	gi|77993368	Acyl-CoA synthetase family member 2	ACSF2	395	2,30E-33	68641	8,39	11	21	-	-	**-1,62**	9,31E-04	**-1,4**	3,80E-03
93	gi|6393848	Trans-2-enoyl-CoA reductase, mitochondrial	MECR	215	2,30E-15	40529	8,92	7	23	-	-	-	-	**-1,58**	0,0371

Carbohydrate metabolism

Spot number[a]	Accession code[b]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		SHR/DM1 vs. C[k,l]	
52	gi|158186649	Enolase 1, (alpha)	ENO1	369	9,20E-31	47440	6,16	15	39	**2,2**	1,15E-03	**3,51**	7,31E-06	**3,52**	1,32E-08
47	gi|158186649	Enolase 1, (alpha)	ENO1	96	0,0019	47440	6,16	6	19	-	-	**1,79**	6,96E-04	**1,35**	0,0387
48	gi|158186649	Enolase 1, (alpha)	ENO1	386	2,00E-32	47440	6,16	13	37	-	-	**1,94**	5,48E-04	**2,02**	1,38E-03
49	gi|158186649	Enolase 1, (alpha)	ENO1	370	7,80E-31	47440	6,16	11	34	-	-	**1,66**	1,35E-03	-	-
74	gi|16757994	Pyruvate kinase, muscle	PKM2	143	4,00E-08	58294	6,63	4	8	**-1,55**	2,37E-03	**-1,46**	0,0137	-	-
61	gi|60688224	PDHX protein	PDHX	105	0,00025	41036	8,83	6	16	-	-	**1,66**	0,0413	-	-
21	gi|158138498	Muscle glycogen phosphorylase	PYGM	698	1,20E-63	97740	6,65	33	46	-	-	**-1,48**	8,94E-05	-	-
70	gi|67078526	UDP-glucose pyrophosphorylase 2	UGP2	147	1,50E-08	57159	7,18	5	9	-	-	-	-	**1,43**	8,30E-03
109	gi|6981146	L-lactate dehydrogenase B	LDBH	211	5,80E-15	36874	5,7	8	27	-	-	-	-	**-1,37**	5,91E-04

Tri-Carboxilic Acid cycle

Spot number[a]	Accession code[b]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		SHR/DM1 vs. C[k,l]	
24	gi|40538860	Aconitase 2, mitochondrial	ACO2	142	5,00E-08	86151	7,87	5	8	-	-	**-1,54**	0,0467	-	-
27	gi|40538860	Aconitase 2, mitochondrial	ACO2	321	6,30E-26	86121	7,87	15	27	**-1,37**	0,0955	**-1,57**	0,0153	-	-
104	gi|148693875	Isocitrate dehydrogenase 3 (NAD+) alpha precursor	IDH3A	121	1,80E-07	40044	6,47	2	6	**-2,09**	0,004	**-2,3**	0,002	-	-
37	gi|21313536	Dihydrolipoamide S-succinyltransferase (E2 comp.)	DLST	105	0,00023	49306	9,11	3	6	-	-	-	-	**-1,54**	0,016
38	gi|21313536	Dihydrolipoamide S-succinyltransferase (E2 comp.)	DLST	88	0,00024	49306	9,11	2	4	-	-	-	-	**-2,05**	0,00154
40	gi|21313536	Dihydrolipoamide S-succinyltransferase (E2 comp.)	DLST	153	1,10E-10	49306	9,11	7	18	**-1,42**	0,0422	**-1,58**	5,67E-03	-	-
85	gi|149036441	Succinate-CoA ligase, GDP-forming, alpha subunit	SUCLG1	158	1,20E-09	37935	9,54	5	16	**-1,73**	0,0164	**-1,53**	6,80E-03	-	-
88	gi|42476181	Malate dehydrogenase, mitochondrial	MDH2	348	1,20E-28	36117	8,93	10	36	**-1,5**	0,0069	**-1,5**	0,0005	-	-
80	gi|18543177	Citrate synthase	CS	124	2,90E-06	52176	8,53	3	7	-	-	-	-	**-1,41**	1,34E-03

Supplementary Table 1.

Mitochondrial Respiratory Chain and Energy Carriers

Spot number[a]	Accession code[b]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		DM1/SHR vs. C[k,l]	
83	gi|149029485	ATP synthase, F1 complex, alpha subunit	ATP5A1	188	1,20E-12	54632	8,24	6	17	**-2,23**	0,000015	**-2,39**	6,9E-06	-	-
67	gi|149029483	ATP synthase, F1 complex, alpha subunit	ATP5A1	258	1,10E-19	54632	8,24	10	27	-	-	-	-	**-1,48**	7,16E-05
78	gi|149029483	ATP synthase, F1 complex, alpha subunit	ATP5A1	419	9,20E-36	54632	8,24	14	36	-	-	-	-	**-1,35**	3,14E-03
117	gi|51948476	Ubiquinol-cytochrome c reductase core protein I	UQCRC1	172	4,90E-11	53500	5,57	4	13	**-2,49**	1,82E-03	**-2,31**	9,96E-04	-	-
100	gi|170295834	NADH dehydrogenase-1 alpha subcomplex 10 prec.	NDUFA10	540	7,80E-48	40753	7,64	25	62	-	-	**-26,12**	7,66E-09	-	-
81	gi|55741424	NADH dehydrogenase flavoprotein 1, 51kDa	NDUFV1	107	0,00016	51383	8,37	7	19	-	-	**1,51**	8,23E-03	-	-
127	gi|52350626	Cyc1 protein	CYC1	198	1,10E-13	34725	9,02	6	21	-	-	-	-	**-1,4**	0,033
125	gi|52350626	Cyc1 protein	CYC1	193	3,60E-13	34725	9,02	4	17	-	-	-	-	**-2,06**	5,10E-04
129	gi|52350626	Cyc1 protein	CYC1	148	1,20E-08	34725	9,02	2	10	-	-	-	-	**-1,49**	2,37E-03
87	gi|38259206	Creatine kinase, mitochondrial 2	CKMT2	254	2,90E-19	47899	8,64	10	24	-	-	-	-	**-1,36**	3,90E-03
89	gi|6671762	Creatine kinase, muscle	CKM	163	3,90E-10	43246	6,58	3	9	**-2**	5,58E-04	**-1,89**	4,64E-04	-	-
115	gi|203476	Creatine kinase, brain, isoform CRA_a	CKB	187	1,50E-12	40883	5,32	5	20	-	-	-	-	**1,38**	0,015
116	gi|31542401	Creatine kinase, brain, isoform CRA_a	CKB	343	3,90E-28	42970	5,33	9	34	-	-	**2,07**	0,00098	**1,65**	2,74E-03

Cytoskeleton regulation

Spot number[a]	Accession code[b]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		SHR/DM1 vs. C[k,l]	
36	gi|38197675	Desmin	DES	690	7,20E-63	53447	5,21	21	51	**1,42**	0,02	**1,53**	0,0005	**1,4**	0,00099
31	gi|84872219	LIM domain binding 3 isoform d	LDB3	107	0,00014	68111	8,28	3	7	-	-	-	-	**2,44**	2,45E-04
29	gi|84872219	LIM domain binding 3 isoform d	LDB3	172	4,60E-11	68111	8,28	7	14	-	-	-	-	**1,6**	0,0036
30	gi|84872219	LIM domain binding 3 isoform d	LDB3	134	3,10E-07	68111	8,28	5	13	**1,47**	0,0686	**1,98**	2,03E-03	-	-
131	gi|13928940	Four and a half LIM domains 2	FHL2	145	2,30E-08	34060	7,31	5	18	-	-	-	-	**2,78**	8,45E-04
97	gi|8393153	PDZ and LIM domain 1	PDLIM	131	6,30E-07	35959	6,56	3	13	**1,62**	1,84E-05	**1,38**	3,82E-03	-	-
141	gi|16924004	Cysteine and glycine-rich protein 3	CSRP3	260	7,80E-20	21701	8,81	8	46	-	-	**2,86**	1,46E-04	**2,19**	4,39E-03
53	gi|211065497	Rho GTPase activating protein 1	ARHGAP1	141	5,80E-08	50648	6,06	2	6	-	-	-	-	**1,65**	9,90E-05
122	gi|149025875	Myozenin 2 isoform CRA_a	MYOZ2	267	1,50E-20	29828	6,99	9	33	-	-	-	-	**1,37**	1,75E-03
108	gi|387090	Alpha-cardiac actin	ACTC1	192	8,90E-15	42043	5,23	5	18	-	-	**-2,27**	0,0343	**-2,13**	0,0328
4	gi|60552352	Myosin heavy chain-6	MYH6	107	0,00016	115591	8,26	8	8	**1,93**	0,111	**1,95**	0,0103	-	-
155	gi|153791853	Myosin, light polypeptide 2, regulatory, cardiac, slow	MYL2	549	9,90E-49	18852	4,86	17	84	-	-	**1,48**	0,0401	-	-

Mitochondrial Respiratory Chain and Energy Carriers

Spot number[a]	Accession code[b]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		SHR/DM1 vs. C[k,l]	
83	gi|149029485	ATP synthase, F1 complex, alpha subunit	ATP5A1	188	1,20E-12	54632	8,24	6	17	**-2,23**	0,000015	**-2,39**	6,9E-06	-	-
67	gi|149029483	ATP synthase, F1 complex, alpha subunit	ATP5A1	258	1,10E-19	54632	8,24	10	27	-	-	-	-	**-1,48**	7,16E-05
78	gi|149029483	ATP synthase, F1 complex, alpha subunit	ATP5A1	419	9,20E-36	54632	8,24	14	36	-	-	-	-	**-1,35**	3,14E-03
117	gi|51948476	Ubiquinol-cytochrome c reductase core protein I	UQCRC1	172	4,90E-11	53500	5,57	4	13	**-2,49**	1,82E-03	**-2,31**	9,96E-04	-	-
100	gi|170295834	NADH dehydrogenase-1 alpha subcomplex 10 prec.	NDUFA10	540	7,80E-48	40753	7,64	25	62	-	-	**-26,12**	7,66E-09	-	-
81	gi|55741424	NADH dehydrogenase flavoprotein 1, 51kDa	NDUFV1	107	0,00016	51383	8,37	7	19	-	-	**1,51**	8,23E-03	-	-
127	gi|52350626	Cyc1 protein	CYC1	198	1,10E-13	34725	9,02	6	21	-	-	-	-	**-1,4**	0,033
125	gi|52350626	Cyc1 protein	CYC1	193	3,60E-13	34725	9,02	4	17	-	-	-	-	**-2,06**	5,10E-04
129	gi|52350626	Cyc1 protein	CYC1	148	1,20E-08	34725	9,02	2	10	-	-	-	-	**-1,49**	2,37E-03
87	gi|38259206	Creatine kinase, mitochondrial 2	CKMT2	254	2,90E-19	47899	8,64	10	24	-	-	-	-	**-1,36**	3,90E-03
89	gi|6671762	Creatine kinase, muscle	CKM	163	3,90E-10	43246	6,58	3	9	**-2**	5,58E-04	**-1,89**	4,64E-04	-	-
115	gi|203476	Creatine kinase, brain, isoform CRA_a	CKB	187	1,50E-12	40883	5,32	5	20	-	-	-	-	**1,38**	0,015
116	gi|31542401	Creatine kinase, brain, isoform CRA_a	CKB	343	3,90E-28	42970	5,33	9	34	-	-	**2,07**	0,00098	**1,65**	2,74E-03

Supplementary Table 1 (cont.).

Miscellneous proteins

Spot number[a]	Accession code[c]	Protein name[c]	HGNC name[d]	Score[e]	Mascot expect.[f]	MW Da, theor.[g]	pI theor.[h]	Matched peptides[i]	Cover. %[j]	Biological Function[m]	Average Vol. and P-value DM1 vs. C[k,l]		SHR vs. C[k,l]		SHR/DM1 vs. C[k,l]	
75	gi|189181716	3-oxoacid CoA transferase 1	OXCT1	161	6,30E-10	56624	8,7	5	12	Involved in ketone body metabolism	-	-	**1.36**	0.00841	-	-
134	gi|13242293	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	HMGCL	146	1,80E-08	34626	8,89	4	12	Involved in ketogenesis from FFAs and Leucine catabolism	-	-	-	-	**1.39**	0.0025
152	gi|2145145	Apolipoprotein A-I	APOA1	157	1,40E-09	29956	5,51	9	27	HDL-apoprotein and involved in spermatozoa motility	-	-	-	-	**2.31**	0.0237
137	gi|20072952	AU RNA binding protein/enoyl CoA reductase	AUH	178	1,20E-11	32906	9,57	6	24	Homologous to enoyl-CoA hidratase (ß-oxidation)	-	-	**1.71**	0.00145	-	-
90	gi|11693174	Branched chain aminotransferase 2, mitochondrial	BCAT2	177	1,60E-11	44817	8,46	9	24	Catabolic enzyme of the esential branched chain amino acids Leu, Isoleu and Val	**-1.61**	0.0354	**-1.35**	0.00169	-	-
91	gi|38512141	Branched chain aminotransferase 2, mitochondrial	BCAT2	206	1,80E-14	44138	8,62	5	13	Catabolic enzyme of the esential branched chain amino acids Leu, Isoleu and Val	-	-	-	-	**-3.44**	0.00138
121	gi|56972641	Clybl protein	CLYBL	169	9,20E-11	37509	8,18	4	22	Similar to citrate lyase beta	-	-	-	-	**1.38**	0.0176
119	gi|78214350	Coenzyme Q9 homolog	COQ9	198	1,10E-13	35295	5,5	3	11	Involved in biosynthesis of coenzyme Q	-	-	-	-	**4.46**	0.00000188
120	gi|78214350	Coenzyme Q9 homolog	COQ9	309	9,90E-25	35295	5,5	5	23	Involved in biosynthesis of coenzyme Q	-	-	**-5.89**	0.0011	**-4.07**	0.005
15	gi|6457378	Cytovillin 2, ezrin	EZR	121	6,20E-06	16294	9,32	3	14	Serves as intermediate between plasma membrane and actin cytoskeleton	**1.71**	0.00693	-	-	-	-
148	gi|149036525	Deoxyguanosine kinase	DGUOK	160	7,30E-10	32296	8,74	5	23	Involved in the phosphorylation of purins	-	-	-	-	**-1.36**	0.00852
62	gi|7106303	EH-domain containing 1	EHD1	540	7,90E-48	60622	6,35	29	46	Involved in IGF-1 receptors endocytosis	-	-	**1.58**	0.000867	-	-
145	gi|148690712	Electron transferring flavoprotein, beta polypeptide	ETFB	95	0,0025	21893	8,85	2	12	Shuttles electrons between ß-oxidation flavoproteins and membrane flavoproteins	-	-	**1.55**	0.0000361	-	-
139	gi|62945328	Glioblastoma amplified sequence	GBAS	318	1,10E-25	33092	9,4	8	20	Amplified in glioblastoma and may be iinvolved in vesicular transport	-	-	**-1.53**	0.000126	**-1.57**	0.00000119
9	gi|71043858	Hydroxysteroid dehydrogenase like 2	HSDL2	213	3,60E-15	58649	5,85	6	18	Hydroxysteroid dehydrogenase/reductase	-	-	-	-	**-2.96**	0.000000468
150	gi|51092266	Hypoxanthine phosphoribosyltransferase 1	HPRT1	149	2,80E-10	24690	6,07	4	22	Involved in the generation of purine nucleotides	**1.52**	0.00741	-	-	-	-
19	gi|57528264	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)	MCCC1	124	3,20E-06	79564	6,66	5	10	Participates in the catalysis of 3-methylcrotonyl-CoA	**3.66**	0.000272	**1.7**	0.00751	-	-
72	gi|58865926	Methylcrotonoyl-Coenzyme A carboxylase 2 (beta)	MCCC2	123	3,70E-06	61992	8,56	7	16	Participates in the catalysis of 3-methylcrotonyl-CoA	-	-	-	-	**1.8**	0.000552
50	gi|3121992	Mitochondrial aldehyde dehydrogenase	ALDH2	185	1,50E-12	56079	6,69	11	28	Mitochondrial dehydrogenase isoform for acetaldehydes	**-1.82**	0.03	**2.03**	0.029	-	-
79	gi|183986052	Monoamino oxidase	MAOA	272	4,60E-21	59393	8,36	6	15	Degrades amine neurotransmiters	-	-	-	-	**1.96**	0.0484
25	gi|183986052	Monoamino oxidase	MAOA	219	9,20E-16	59393	8,36	7	17	Degrades amine neurotransmiters	-	-	-	-	**1.78**	0.0314
126	gi|77628000	Nitrilase family, member 2	NIT2	178	3,50E-13	31024	6,9	7	26	Arrests cultured cells in G2 phase of the cell cycle	-	-	**-1.66**	0.00151	-	-
2	gi|37812499	Optic atrophy 1-like protein	OPA1	247	1,50E-18	111737	7,17	12	15	GTPase required for mitochondrial fusion and apoptosis regulation	-	-	-	-	**-1.35**	0.00702
138	gi|8393948	Phosphoglycerate mutase 2	PGAM2	133	4,00E-07	28908	8,85	7	30	Interconversion of 3- and 2-P-Glycerate and 2,3-biP-Glycerate (Glycolysis)	**-1.69**	0.00105	**-1.35**	0.0256	-	-
41	gi|13929024	Protein phosphatase 5, catalytic subunit	PPP5C	163	3,60E-10	57507	5,84	6	14	May play a role in the regulation of RNA biogenesis and/or mitosis	-	-	-	-	**2.16**	0.0000952
149	gi|162329549	SCO cytochrome oxidase deficient homolog 2	SCO2	101	0,00058	29097	8,41	2	13	Involved in the citochrome oxidase synthesis	-	-	-	-	**1.53**	0.00507
42	gi|294639	Sulfite oxidase	SUOX	107	0,00014	54606	5,79	2	5	Catalyzes the oxidation of sulfite to sulfate from the amino acids Cys and Met	-	-	-	-	**1.89**	0.00376
60	gi|556395	Succinate semialdehyde dehydrogenase	ALDH5A1	202	5,00E-14	52669	6,4	11	26	Mitochondrial succinic semialdehyde dehydrogenase	-	-	**-1.47**	0.00303	-	-
147	gi|38512111	Triophosphate isomerase 1	TPI1	207	1,40E-14	27214	7,07	10	49	Isomerase for the conversión of G3P to DHAP in glycolysis and gluconeogenesis	-	-	-	-	**1.37**	0.000719
112	gi|149025115	Zinc binding alcohol dehydrogenase, domain 1	ZADH1	137	1,60E-07	14101	5,72	1*	12	Involved in the prostaglandins metabolism and activation of the PPAR	**3.29**	0.00736	**3.5**	0.00268	-	-

Supplementary Table 1 (cont.2).

Supplemental Table 1. Proteins identified by MALDI-MS showing significant changes in the long-term DM1 and hypertensive model. Proteins were classified into the following categories: ß-oxidation, carbohydrate, TCA-cycle enzymes, mitochondrial respiratory chain, energy carriers, cytoskeleton, apoptosis and miscellaneous. [a]Spot numbering as in Figure 1B and 2. [b]Protein accession code from the NCBInr database; [c]Protein name; [d]HGNC acronym; [e]Mascot score; [f]Expected value; [g]Theoretical molecular weight (kDa); [h]Isoelectric point; [i]Number of matched peptides; [j]Protein sequence coverage for the most probable candidate as provided by Mascot; [k]Average volumen ratio (for DM1, SHR, SHR/DM1 vs. control) as calculated by the DeCyder BVA analysis; [l]Student t-test P-value; and [m]Biological function retrieved from SwissProt. *See supplemental data.

Suplemental Data

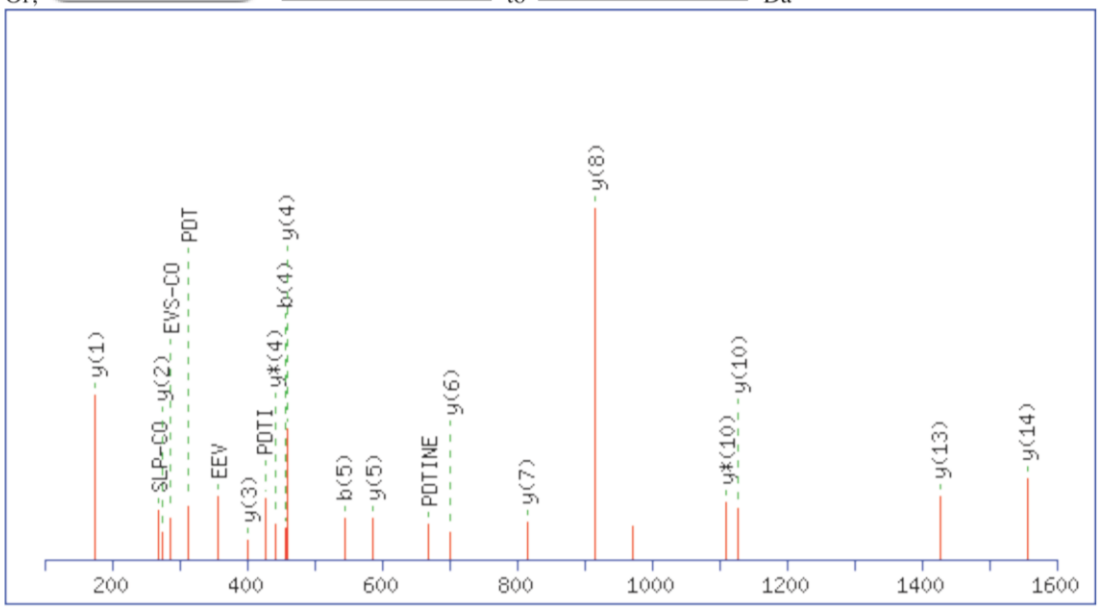
Zinc binding alcohol dehydrogenase, domain 1 (ZADH1).
Due to ZADH1 was matched only by one peptide, we show its spectra:

Mascot Search Results

Peptide View

MS/MS Fragmentation of **VEEVSLPDTINEGQVR**
Found in **gi|149025115**, zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_a [Rattus norvegicus]
Match to Query 2: 1783.887996 from(1784.895272,1+)
Data file DATA.TXT

Or, Plot from 100 to 1600 Da

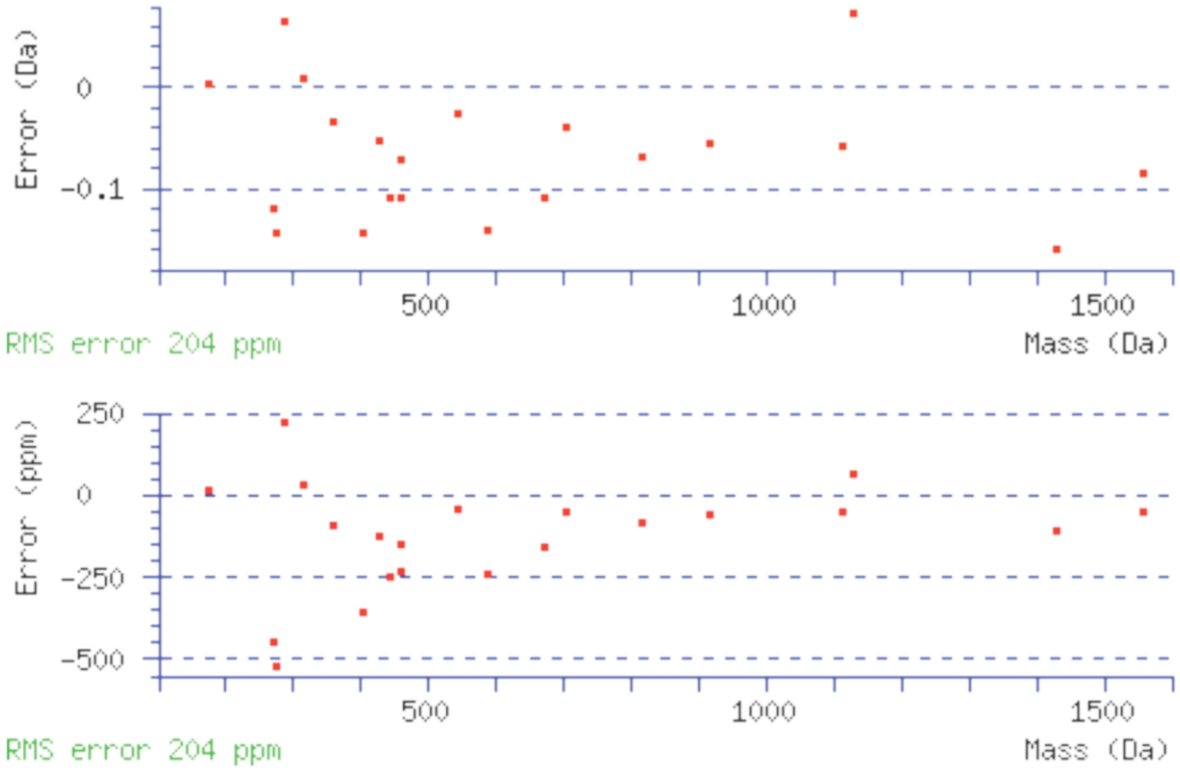


Monoisotopic mass of neutral peptide Mr(calc): 1783.8952
Fixed modifications: Carbamidomethyl (C)
Ions Score: 115 **Expect:** 6.9e-09
Matches (Bold Red): 23/267 fragment ions using 22 most intense peaks

#	Immon.	a	a*	a^0	b	b*	b^0	Seq.	v	w	w'	y	y*	y^0	#
1	72.0808	72.0808			100.0757			V							16
2	102.0550	201.1234		183.1128	229.1183		211.1077	E	1611.7973	1610.8020		1685.8341	1668.8075	1667.8235	15
3	102.0550	330.1660		312.1554	**358.1609**		340.1503	E	1482.7547	1481.7594		**1556.7915**	1539.7649	1538.7809	14
4	72.0808	429.2344		411.2238	**457.2293**		439.2187	V	1383.6863	1396.7067		**1427.7489**	1410.7223	1409.7383	13
5	60.0444	516.2664		498.2558	**544.2613**		526.2508	S	1296.6543	1295.6590		1328.6805	1311.6539	1310.6699	12
6	86.0964	629.3505		611.3399	657.3454		639.3348	L	1183.5702	1182.5749		1241.6484	1224.6219	1223.6379	11
7	70.0651	726.4032		708.3927	754.3981		736.3876	P	1086.5174	1085.5222		**1128.5644**	**1111.5378**	1110.5538	10
8	88.0393	841.4302		823.4196	869.4251		851.4145	D	971.4905	970.4952		1031.5116	1014.4851	1013.5010	9
9	74.0600	942.4779		924.4673	970.4728		952.4622	T	870.4428	883.4632	885.4425	**916.4847**	899.4581	898.4741	8
10	86.0964	1055.5619		1037.5514	1083.5568		1065.5463	I	757.3587	770.3791	784.3948	**815.4370**	798.4104	797.4264	7
11	87.0553	1169.6048	1152.5783	1151.5943	1197.5998	1180.5732	1179.5892	N	643.3158	642.3206		**702.3529**	685.3264	684.3424	6
12	102.0550	1298.6474	1281.6209	1280.6369	1326.6424	1309.6158	1308.6318	E	514.2732	513.2780		**588.3100**	571.2835	570.2994	5
13	30.0338	1355.6689	1338.6424	1337.6583	1383.6638	1366.6373	1365.6533	G				**459.2674**	**442.2409**		4
14	101.0709	1483.7275	1466.7009	1465.7169	1511.7224	1494.6958	1493.7118	Q	329.1932	328.1979		**402.2459**	385.2194		3
15	72.0808	1582.7959	1565.7693	1564.7853	1610.7908	1593.7643	1592.7802	V	230.1248	243.1452		**274.1874**	257.1608		2
16	129.1135							R	74.0237	73.0284		**175.1190**	158.0924		1

Seq	ya	yb	Seq	ya	yb	Seq	ya	yb
EE	231.0975	259.0925	EEV	330.1660	**358.1609**	EEVS	417.1980	445.1929
EEVSL	530.2821	558.2770	EEVSLP	627.3348	655.3297	EV	201.1234	229.1183
EVS	**288.1554**	316.1503	EVSL	401.2395	429.2344	EVSLP	498.2922	526.2871
EVSLPD	613.3192	641.3141	VS	159.1128	187.1077	VSL	272.1969	300.1918
VSLP	369.2496	397.2445	VSLPD	484.2766	512.2715	VSLPDT	585.3243	613.3192
VSLPDTI	698.4083	726.4032	SL	173.1285	201.1234	SLP	**270.1812**	298.1761
SLPD	385.2082	413.2031	SLPDT	486.2558	514.2508	SLPDTI	599.3399	627.3348
LP	183.1492	211.1441	LPD	298.1761	326.1710	LPDT	399.2238	**427.2187**
LPDTI	512.3079	540.3028	LPDTIN	626.3508	654.3457	PD	185.0921	213.0870
PDT	286.1397	**314.1347**	PDTI	399.2238	**427.2187**	PDTIN	513.2667	541.2617
PDTINE	642.3093	**670.3042**	PDTINEG	699.3308	727.3257	DT	189.0870	217.0819

DTI	302.1710	330.1660	**DTIN**	416.2140	444.2089	**DTINE**	545.2566	573.2515
DTINEG	602.2780	630.2729	**TI**	187.1441	215.1390	**TIN**	301.1870	329.1819
TINE	430.2296	458.2245	**TINEG**	487.2511	515.2460	**TINEGQ**	615.3097	643.3046
IN	200.1394	228.1343	**INE**	329.1819	357.1769	**INEG**	386.2034	414.1983
INEGQ	514.2620	542.2569	**INEGQV**	613.3304	641.3253	**NE**	216.0979	244.0928
NEG	273.1193	301.1143	**NEGQ**	401.1779	429.1728	**NEGQV**	500.2463	528.2413
EG	159.0764	187.0713	**EGQ**	287.1350	315.1299	**EGQV**	386.2034	414.1983
GQ	158.0924	186.0873	**GQV**	257.1608	285.1557	**QV**	200.1394	228.1343



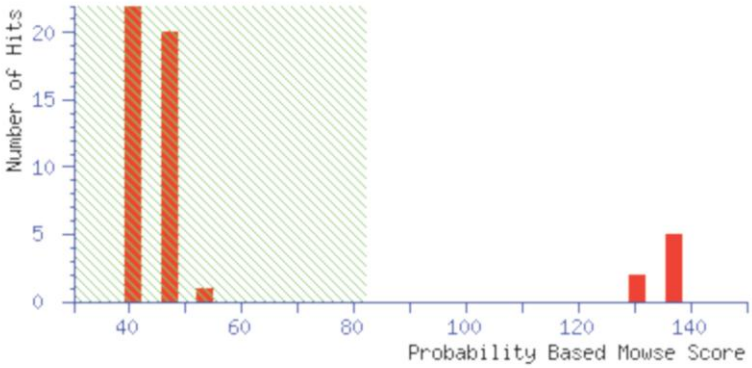
NCBI **BLAST** search of VEEVSLPDTINEGQVR
(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)
Other BLAST web gateways

Mascot Search Results

```
User            : emi
 Email          : proteomics.maldi@cnic.es
 Search title   :
 MS data file   : DATA.TXT
 Database       : NCBInr 20090306 (7969746 sequences; 2747354109 residues)
 Timestamp      : 11 Mar 2009 at 15:44:33 GMT
 Warning        : A Peptide summary report will usually give a much clearer picture of MS/MS search
results.
 Top Score      : 137 for gi|149025115, zinc binding alcohol dehydrogenase, domain containing 1, isoform
CRA_a [Rattus norvegicus]
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 82 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein Summary Report

		Help
Significance threshold p< 0.05	Max. number of hits 5	
Standard scoring ◉ MudPIT scoring ○	Ions score or expect cut-off 0	Show sub-sets 0
Show pop-ups ◉ Suppress pop-ups ○	Sort unassigned	Require bold red ☐ (hidden)

Index

	Accession	Mass	Score	Description
1.	gi|149025115	14101	137	zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_a [Rattus norvegicus]
2.	gi|149025120	19424	136	zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_e [Rattus norvegicus]
3.	gi|149025122	22678	135	zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_g [Rattus norvegicus]
4.	gi|149025123	25327	135	zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_h [Rattus norvegicus]
5.	gi|62543513	30220	134	prostaglandin reductase 2 [Rattus norvegicus]

Results List

1. gi|149025115 **Mass:** 14101 **Score:** 137 **Expect:** 1.6e-07 **Queries matched:** 1
zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_a [Rattus norvegicus]

Observed	Mr(expt)	Mr(calc)	ppm	Start		End	Miss	Ions	Peptide
1784.8953	1783.8880	1783.8952	-4.04	25	-	40	0	115	R.VEEVSLPDTINEGQVR.V

No match to: 1507.7351

2. gi|149025120 **Mass:** 19424 **Score:** 136 **Expect:** 2e-07 **Queries matched:** 1
zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_e [Rattus norvegicus]

Observed	Mr(expt)	Mr(calc)	ppm	Start		End	Miss	Ions	Peptide
1784.8953	1783.8880	1783.8952	-4.04	25	-	40	0	115	R.VEEVSLPDTINEGQVR.V

No match to: 1507.7351

3. gi|149025122 **Mass:** 22678 **Score:** 135 **Expect:** 2.5e-07 **Queries matched:** 1
zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_g [Rattus norvegicus]

Observed	Mr(expt)	Mr(calc)	ppm	Start		End	Miss	Ions	Peptide
1784.8953	1783.8880	1783.8952	-4.04	25	-	40	0	115	R.VEEVSLPDTINEGQVR.V

No match to: 1507.7351

4. gi|149025123 **Mass:** 25327 **Score:** 135 **Expect:** 2.5e-07 **Queries matched:** 1
zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_h [Rattus norvegicus]

Observed	Mr(expt)	Mr(calc)	ppm	Start		End	Miss	Ions	Peptide
1784.8953	1783.8880	1783.8952	-4.04	25	-	40	0	115	R.VEEVSLPDTINEGQVR.V

No match to: 1507.7351

5. gi|62543513 **Mass:** 30220 **Score:** 134 **Expect:** 3.2e-07 **Queries matched:** 1
prostaglandin reductase 2 [Rattus norvegicus]

Observed	Mr(expt)	Mr(calc)	ppm	Start		End	Miss	Ions	Peptide
1784.8953	1783.8880	1783.8952	-4.04	25	-	40	0	115	R.VEEVSLPDTINEGQVR.V

No match to: 1507.7351

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.7 Da
Max Missed Cleavages : 1
Instrument type : MALDI-TOF-TOF
Query1 (1507.7351,1+) : <no title>
Query2 (1784.8953,1+) : <no title>

Mascot Search Results

Protein View

Match to: **gi|149025115** Score: **137** Expect: **1.6e-07**
zinc binding alcohol dehydrogenase, domain containing 1, isoform CRA_a [Rattus norvegicus]
Found in search of DATA.TXT

Nominal mass (M_r): **14101**; Calculated pI value: **5.72**
NCBI BLAST search of gi|149025115 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Rattus norvegicus
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|149025119 from Rattus norvegicus

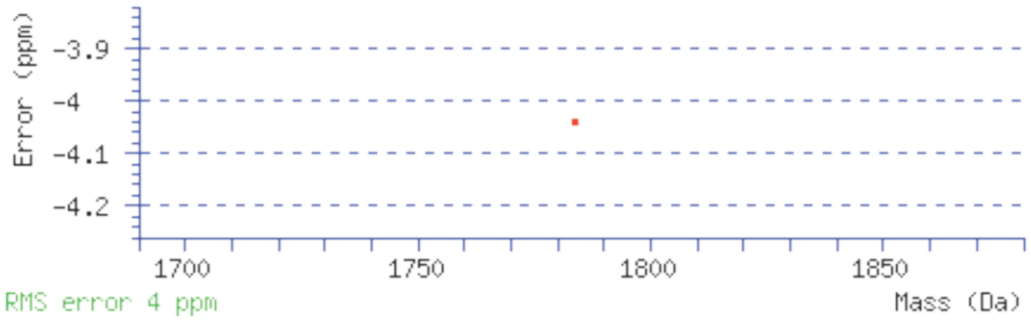
Fixed modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **12%**

Matched peptides shown in **Bold Red**

```
     1 MIIQRVVLDS RPGKNGNPVA ENFRVEEVSL PDTINEGQVR VRTLYLSVDP
    51 YMRCKMNEET GADYLAPWCS LAGGSLPLGQ ALRVHSLILQ FPFLFLLGAE
   101 GVISISCSCH QPPPHPTPYP LHDGLL
```

Sort Peptides By — Residue Number | Increasing Mass | Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
25 - 40	1784.8953	1783.8880	1783.8952	-4	0	R.VEEVSLPDTINEGQVR.V (Ions score 115)



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LOCUS       EDL81482                126 aa            linear   ROD 20-JUN-2007
DEFINITION  zinc binding alcohol dehydrogenase, domain containing 1, isoform
            CRA_a [Rattus norvegicus].
ACCESSION   EDL81482
VERSION     EDL81482.1  GI:149025115
DBSOURCE    accession CH473982.1
KEYWORDS    .
SOURCE      Rattus norvegicus (Norway rat)
  ORGANISM  Rattus norvegicus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Rattus.
REFERENCE   1  (residues 1 to 126)
  AUTHORS   Florea,L., Di Francesco,V., Miller,J., Turner,R., Yao,A.,
            Harris,M., Walenz,B., Mobarry,C., Merkulov,G.V., Charlab,R.,
            Dew,I., Deng,Z., Istrail,S., Li,P. and Sutton,G.
  TITLE     Gene and alternative splicing annotation with AIR
  JOURNAL   Genome Res. 15 (1), 54-66 (2005)
   PUBMED   15632090
REFERENCE   2  (residues 1 to 126)
  AUTHORS   Mural,R.J., Li,P.W., Adams,M.D., Amanatides,P.G., Baden-Tillson,H.,
            Barnstead,M., Chin,S.H., Dew,I., Evans,C.A., Ferriera,S.,
```

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            Flanigan,M., Fosler,C., Glodek,A., Gu,Z., Holt,R.A., Jennings,D.,
            Kraft,C.L., Lu,F., Nguyen,T., Nusskern,D.R., Pfannkoch,C.M.,
            Sitter,C., Sutton,G.G., Venter,J.C., Wang,Z., Woodage,T.,
            Zheng,X.H. and Zhong,F.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-JUL-2005) Celera Genomics, 45 W. Gude Dr., Rockville,
            MD 20850, USA
COMMENT     Method: conceptual translation.
FEATURES             Location/Qualifiers
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                     /strain="BN; Sprague-Dawley"
                     /db_xref="taxon:10116"
                     /chromosome="6"
     Protein         1..126
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                     containing 1, isoform CRA_a"
     Region          2..>68
                     /region_name="COG2130"
                     /note="Putative NADP-dependent oxidoreductases [General
                     function prediction only]; COG2130"
                     /db_xref="CDD:32313"
     CDS             1..126
                     /gene="Zadh1"
                     /locus_tag="rCG_20970"
                     /coded_by="join(CH473982.1:3896506..3896542,
                     CH473982.1:3898607..3898725,CH473982.1:3901223..3901266,
                     CH473982.1:3907813..3907993)"
                     /note="gene_id=rCG20970.0 transcript_id=rCT20962.0
                     protein_id=rCP14918.0 isoform=CRA_a"
                     /db_xref="GeneID:299194"
```

3. APOPTOSIS CARDIACA EN UN MODELO EXPERIMENTAL DE DIABETES TIPO II Y OBESIDAD. EFECTOS PROTECTORES DE LA EPLERENONA.

En DM, la asociación con obesidad puede dar lugar a efectos dañinos adicionales en el corazón. Sin embargo no se conocen los mediadores ni los mecanismos moleculares subyacentes. El exceso de ácidos grasos y lipoproteínas circulantes puede resultar en un inadecuado almacenamiento y metabolismo de ácidos grasos, que podría inducir apoptosis cardiaca[148,45]. El SRAA local podría estar activado y participar en el desarrollo de la patología DM2-obesa. Los bloqueantes del SRAA, basados en la inhibición de los receptores de AngII, reducen la disfunción diastólica y apoptosis en pacientes diabéticos asintomáticos[10]. Sin embargo, dado los efectos pleiotrópicos de la AngII, un efector posterior en la vía como es la aldosterona, podría constituir una diana terapéutica alternativa. La aldosterona induce respuestas apoptóticas a través de mecanismos dependientes de receptores de muerte y daño mitocondrial[27]. Estos efectos pueden enfatizados con la presencia de obesidad[62]. Hasta la fecha se ha demostrado que la eplerenona induce acciones anti-fibróticas y anti-apoptóticas en la hipertrofia ventricular izquierda, hipertensión e infarto de miocardio[10,133,135]. Sin embargo no se conocen sus acciones en el corazón diabético. En nuestros estudios, el miocardio diabético-obeso presentó disfunción cardiaca e incremento de la apoptosis con activación de la vía apoptótica Fas-caspasa 8-caspasa 3. Interesantemente, el tratamiento con eplerenona mejoró estos parámetros. Además, en cardiomiocitos en cultivo la alta concentración de ácido graso saturado produjo apoptosis mediante mecanismos independientes de Fas, aunque eplerenona también mitigó esta respuesta.

Title: **Cardiac apoptosis in experimental obese type-II diabetes. Protective effects of eplerenone.**

Authors: S. Ares-Carrasco[1,3], E. Ramírez[1,3], B. Picatoste[1,3], A. Caro-Vadillo[2,3], J. Egido[1,3], J. Tuñón[1,3*] and Ó. Lorenzo[1,3*].

[1]IIS-Fundación Jiménez Díaz, Madrid.

[2]Veterinary School, Complutense University, Madrid

[3]This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation

Conflict of interest: None declared

Acknowledgments: We would like to thank Mr Oliver Shaw for his excellent word processing. This work was supported by national grants from Ministerio de Educación y Ciencia (SAF2009-08367), Comunidad de Madrid (CCG10-UAM/BIO-5289), and a grant from by Pfizer (NY, USA).

Corresponding author:

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Av. Reyes Católicos 2. Madrid 28040, Spain

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Keywords: diabetic cardiomyopathy, eplerenone, apoptosis, steatosis, PPAR

Abstract:

Background: Cardiac steatosis and apoptosis are key processes in diabetic cardiomyopathy, but the underlying cellular mechanisms have not been elucidated, leading to a lack of effective therapy. The aldosterone receptor blocker, eplerenone, has demonstrated anti-fibrotic effects in the diabetic heart.

Objective: To study the role of eplerenone in cardiac steatosis and apoptosis associated to experimental obese/type-II diabetes.

Methods: Non-hypertensive Zucker Diabetic Fatty (ZDF) rats received eplerenone (25 mg/kg) or vehicle. Zucker Lean (ZL) rats were used as control (n=10, each group). After 16 weeks, cardiac structure and function was examined, and plasma and hearts were isolated for biochemical and histological approaches. Cultured cardiomyocytes were used for *in vitro* assays.

Results: In contrast to ZL, ZDF rats exhibited hyperglycemia, hyperaldosteronemia and hyperlipidemia. ZDF hearts showed Fas-system activation, cell apoptosis and hypertrophy. Importantly, eplerenone attenuated these changes. In cultured cardiomyocytes, high-concentrations of palmitate promoted apoptosis by Fas-system independent mechanisms. Interestingly, these events were mitigated by eplerenone.

Conclusions: Experimental obese/type-II diabetes induced myocardial apoptosis and hypertrophy. However, eplerenone attenuated these responses by inhibition of FFA and aldosterone-associated pro-apoptotic actions.

Introduction:

Type-II diabetes is an increasingly prevalent worldwide disease. Heart failure in type-II diabetic patients even in the absence of coronary artery disease or hypertension is a common an entity known as diabetic cardiomyopathy (DCM). DCM is characterized by myocardial apoptosis, and subsequent remodelling fibrosis and hypertrophy [1]. In addition, diverse comorbidities commonly present in diabetes, such as obesity, may accentuate these deleterious responses. The excess of circulating FFA may result in increased cardiac free fatty acid (FFA) uptake, inadequate storage and metabolism, and consequent lipotoxicity and apoptosis [2] [3]. However, the related molecular mechanisms have been poorly investigated, leading to a lack of an effective therapy. In this sense, the renin-angiotensin-aldosterone system (RAAS) is a major homeostatic regulator of cardiac function that participates in the pathogenesis of myocardial diseases such as type-II diabetes [4] [5]. Local RAAS activation has been associated with some hallmarks of the DCM, including fibrosis and apoptosis [6]. In fact, RAAS blockers based on angiotensin-II receptor inhibition improved fibrosis and echocardiographic parameters of diastolic dysfunction in asymptomatic diabetic patients [1]. However, given the pleiotropic role of angiotensin-II the downstream RAAS effector aldosterone may be considered as an alternative target. In fact, aldosterone promoted angiotensin-II actions and fibrosis in the diabetic myocardium by up-regulation of pro-fibrotic/oxidative mediators [7]. Aldosterone also exerted apoptotic responses by both mitochondrial- and death receptors-dependent mechanisms [8] [9] [10] [11], and these effects could be worsened by hyperlipidemia and obesity [1] [6]. Thus, eplerenone, a specific aldosterone

receptor blocker, has demonstrated anti-fibrotic and anti-apoptotic properties in left ventricular hypertrophy, hypertension, and myocardial infarct [7] [12]. Also, in controlled randomized clinical trials, eplerenone reduced mortality in patients with heart failure, independently of hypertension improvement and on top of angiotensin-II inhibition [13]. However, eplerenone actions on DCM and its related molecular mechanisms, particularly in apoptosis, have not been elucidated.

Methods:

Animal model

An obese non-hypertensive model of type-II diabetes was used for this study. Under fatty diet intake (Purina #5008; 26.8% protein, 16.7% fat and 56.4% carbohydrates), Zucker Diabetic Fatty (ZDF) rats closely mimic human adult onset of type-II diabetes and its related complications due to the inherited homozygous leptin receptor mutation (*fa*/*fa*), which leads to obesity and insulin resistance [14]. Obesity and type-II diabetes do not progress in Zucker lean (ZL) littermates fed with standard diet [14]. Male rats (n=10, each group) were purchased from Charles Rivers (France) and were kept on an artificial 12-hour light-dark cycle (7 a.m.-7 p.m.) at 25°C. Rats received tap water *ad libitum*. After full onset of type-II diabetes (at the 14^{th} week), ZDF rats were randomized and received eplerenone [Pfizer; 25 mg/kg/day in drinking water, by oral gavage (10 a.m.)] or vehicle. After 16 weeks of treatment, plasma (collected from cava vein) and hearts were isolated under isoflurane (1.5% in O_2) anaesthesia (3-7 p.m.). Plasma lipid profile, aldosterone, glucose, hepatic enzymes, ions and renal parameters were enzymatically measured in the Clinical Department of the Hospital. Hearts were rinsed, dried and weighted. Some ventricular slices were embedded in p-formaldehyde (to paraffin inclusion) or optimal-cutting-temperature (OCT) compound, for histology. Left ventricles were frozen in liquid-N_2 for biochemical experiments. Body weight and systolic blood pressure (by tail-cuff method) were periodically evaluated. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology, ref.: Coats AJS and Shewan LG. Statement on Authorship and Publishing Ethics (*Int J Cardiol* 2011; 153: 239-40).

Cardiac structure and function

Cardiac echocardiography was performed under 1.5% isoflurane-O_2 anaesthesia in all rats before (not shown) and after the treatment. Both M-mode and two-dimensional (2D) echocardiograms were obtained using a 12 MHz ultra-band sector transducer (En Visor-C-HD, Philips). Images were obtained from the left and right parasternal window in a supine decubitus position. The following parameters were measured and calculated from M-mode tracing: left ventricular (LV) end-diastolic diameter (LVDD), LV end-systolic diameter (LVSD) and ejection fraction (EF). Wall thickness of four segments [anterior, inter-ventricular-septum (IVS), lateral, and posterior (LVPW) walls] was evaluated on short axis 2D images.

Examination of cardiac fibrosis, steatosis, apoptosis and oxidative stress

Paraffin sections (4 μm) of all myocardia were fixed on slides and used for histology. Masson trichrome (Bio-Optica, Milan, Italy) staining was used to determine cardiac fibrosis. The semi-quantitative scores were achieved on ten fields of each myocardium using the Metamorph software. Apoptosis was detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based CardioTACS In Situ Apoptosis Detection Kit (R&D systems; Minneapolis, USA). *In situ* DNA fragmentation was identified by incorporating labeled nucleotides onto the free 3'-OH ends of DNA fragments and using a terminal deoxynucleotidyl transferase enzyme. DNA was visualized by binding streptavidin-horseradish peroxidase followed by reaction with TACS Blue Label™, which generated a dark blue precipitate. The percentage of TUNEL-

positive nuclei relative to total nuclei was determined in a blinded manner by counting 200-300 cells on ten randomly chosen fields per coverslip for each myocardium.

Cultured cardiomyocytes

H9c2(2-1) is a permanent myoblast cell line derived from embryonic BD1X rat heart tissue (ATCC; USA). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 IE/ml sodium penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 5 mM D-glucose (Sigma). Properties of H9c2 cells are similar to those in adult cardiomyocytes. H9c2 differentiated from mononucleated myoblasts into myocytes upon overnight reduction of serum concentration before stimulation. The hyperlipidemic or hyperglycemic conditions were mimicked by incubation with high concentration of saturated (C16:0) FFA palmitate [sodium palmitate (PA), Sigma] or D-glucose (HG), respectively. PA was previously conjugated with BSA in a 3:1 molar ratio as published elsewhere [15]. In control cells, BSA was added as described but in the absence of PA. Eplerenone [1 mM-1 µM; Pfizer] was added 1h before stimulation. Fas and Caspase-8 inhibitors: IL1ß (0.01 ng/ml; Peprotech) and Z-IETD-FMK [Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone, 40 µM; Sigma], respectively, were added 30 min before stimulation.

Detection of apoptosis and survival in cardiomyocytes

Apoptosis was quantified by flow cytometry of cell DNA content. After stimulations, H9c2 were harvested, permeabilized in PBS-0.05% NP-40 and 10

µg/ml RNAse-A (3h, 4°C), and DNA-stained with 100 µg/ml propidium iodide. Lower DNA content (hypodiploid cells; Sub-G0/G1 phase) due to nuclear fragmentation characterized apoptotic cells. The percentage of apoptotic cells with decreased DNA content is shown. To assess the morphological nuclear changes seen in apoptosis, cells were cultured in chamber slides (Nunc; Naperville, IL), stimulated, fixed with methanol:acetone (1:1) and nuclear-stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Condensed, piknotic and fragmented nuclei of apoptotic cells were identified by a laser scanning confocal microscope (Leyka). Cell survival was achieved with a MTT Cell Growth Assay Kit. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] is cleaved by living cells to yield a dark blue formazan product detected by a colorimetric assay ($Abs_{540\ nm}$). Bioreduction of MTT involves endoplasmic reticulum, cytosolic and plasma membranes reductases [16]. MTT was added to cells at a concentration of 0.5 mg/ml for 3h at 37°C, and the number of cells in the bottom of each plate exhibiting a positive blue granular reaction product was quantified.

Western Blot

A piece (50 mg) of homogenized ventricle (Bullet Blender, Cultek) or cell extract (after 14h-stimulation) were dissolved in cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 2% SDS + 1/250 mammalian protease inhibitors). Equal amounts of proteins (20-30 µg) were separated on polyacrylamide gels, transferred to membranes and probed with specific antibodies [anti-FasL, -FAS, -FADD, (Sta. Cruz Biotech.), -caspase-8, and -caspase-3 (Cell Signalling)]. Anti-GAPDH or α-tubulin (Millipore, MA, USA) was used as loading control.

Then, secondary HRP-linked antibodies (GE Healthcare) were used for chemo-luminescence development. For membrane-cytosol separation, cells were suspended in cold lysis buffer (3 mM Imidazole, 250 mM sucrose and 1/250 mammalian protease inhibitors; pH to 7.2) and broken by passing 5 times through a 22g needle. Nuclei were pellet (10' at 3,000 rpm) and post nuclear supernatants were centrifuged again (15' at 65,000 rpm). Supernatants were saved as cytosolic fractions and tubes were aspirated for residual cytosol. The pellet was dissolved in cold lysis buffer as membrane fraction. A representative gel of all rats or at least three independent experiments of cultured cells with the semi-quantification scores (n-fold vs. GAPDH or α-tubulin) are shown.

Statistical analysis

Data are expressed as mean±standard deviation. Multiple comparisons were performed by non-parametric Kruskal-Wallis test followed by a Mann-Whitney test. A two-tailed $p<0.05$ was considered significant.

Results

Reduction of hyperlipidemia by eplerenone in obese/type-II diabetic rats

After 30 weeks, ZDF rats exhibited high plasma levels of aldosterone (430±192.6 vs. 180±57.1 pg/ml ZL rats), glucose (666.6±102.6 vs. 194.5±38.2 mg/dl) and lipid profile [cholesterol (Ch), 187.1±14.1 vs. 61.1±8.3 mg/dl; triglycerides (TG), 397.6±166.8 vs. 39.6±8.3 mg/dl, non-esterified fatty acid (NEFA), 5.1±1.1 vs. 1.6±1.1 mM and HDL, 76.5±10.3 vs. 19.7±2.3 mg/dl] (figure 1A). As expected, proteinuria was also elevated in ZDF rats (134.6±43.1 vs. 8.41±3.5 mg/day). Interestingly, 16 weeks of eplerenone substantially triggered plasma aldosterone (816±154.49 pg/ml) and reduced plasma lipids (Ch, 143.4±12.2 mg/dl; TG, 111.7±33.2 mg/dl and NEFA, 0.73±0.3 mM) and proteinuria (87.6±7.7 mg/day). No significant changes in systolic blood pressure (135.0±8.1 vs. 139.8±15.2 mm Hg ZDF rats) were observed, and markers of severe renal (urea, blood urea nitrogen, creatin and albumin) and liver (ASAT and ALAT) injury remained within the normal ranges in all groups (not shown). Of note, since mineralocorticoid antagonists may induce hyperkalemia [17], we monitored the plasma potassium concentrations. Conveniently, at this dose/time of eplerenone, potassium was kept within the non-toxic levels in all rats (4.4±0.58 mEq/l).

Attenuation of cardiac hypertrophy by eplerenone in ZDF rats

As previously documented [18], at this stage of the disease ZDF rats exhibited weight loss. However, a significant elevation of the heart weight/femur length (HW/FL, 0.5±0.06-fold vs. 0.4±0.03 ZL rats) ratio was observed (figure 1A). In addition, by Echo-Doppler (figure 1B), ZDF hearts exhibited an increase

of the IVS (0.24±0.01 vs. 0.18±0.02 cm ZL myocardium), and a reduction of LVDD (0.51±0.11 vs. 0.71±0.1 cm ZL) and LVSD (0.21±0.07 vs. 0.32±0.09 cm ZL hearts), probably related to IVS hypertrophy. The ejection fraction (EF) was unchanged. Interestingly, HW/FL and IVS were attenuated by eplerenone administration (0.4±0.01-fold and 0.2±0.03 cm, respectively).

Eplerenone ameliorated fibrosis and apoptosis in the ZDF myocardium. Participation of the pro-apoptotic Fas-caspase-8-caspase-3 axis.

Left ventricular myocardium in the ZL rats exhibited a normal architecture with regular interstitial space (figure 2A, top). However, abnormal myocardial architecture (cardiomyocyte hypertrophy and disarray, and enlarged interstitial space) was observed in the ZDF group. This animals exhibited an increase of interstitial and perivascular extra-cellular matrix (ECM) deposition. Notoriously, eplerenone mitigated ECM accumulation, confirming previous data in experimental [19] and human [20] diabetic hearts.

Since fibrosis and hypertrophy contribute to myocardial remodelling in response to a loss of cells [6] [21], we focused in the apoptotic response of ZDF hearts. By TUNEL (figure 2A, bottom), we observed an increase of apoptotic-positive nuclei in ZDF (4.1 vs. 1.2 cells/mm^2 ZL), which was diminished by eplerenone. In addition, a TNFα-superfamily member, FasL, was found elevated in ZDF myocardium (figure 2B). In concordance, the FasL specific receptor, Fas, and the Fas-linked mediator, FADD, were also induced in ZDF (figure 2C). Other pro-apoptotic inducers such as TNFα and TWEAK were not altered (not shown). Interestingly, FasL, Fas and FADD were reduced in the myocardia after eplerenone administration (figure 2B, C). Downstream, Fas-

system associated caspases, caspase-8 and caspase-3, were also activated (cleaved) in the ZDF heart and attenuated by eplerenone (figure 2B, D).

Eplerenone decreased apoptosis by Fas-independent mechanisms in palmitate-induced cardiomyocytes

Cardiac apoptosis in obese/type-II diabetic hearts may result from an excess of plasma FFA and tissue deposition [3][21]. We tested in cardiomyocytes the apoptotic effect of high concentrations of palmitate (PA). PA was added with/without high doses of D-glucose (HG), as hyperglycemic stimulus. PA, but not HG, significantly increased the number of apoptotic cells as early as 14h incubation and at 0.12 mM (figure 3A), similarly to a lethal cytokine (30 U/ml IFNγ; not shown). Of note, co-incubation of PA and HG did not significantly alter the magnitude of PA-induced apoptosis (figure 3B). In addition, caspase-3 activation (figure 3C) and subsequent nuclear pyknosis and cell loss (figure 3D) were detected mostly in PA-incubated cardiomyocytes. However, eplerenone (1 μM) reduced PA-induced apoptosis and caspase-3 activation (figure 4A, B). These data were confirmed by MTT survival assays. Incubation with PA significantly decreased the cardiomyocytes viability and this effect was mitigated by eplerenone (only for PA 0.12 mM) (figure 4C). Intriguingly, in contrast to ZDF heart, FasL, Fas, FADD and caspase-8 expression were not increased after 14h of PA-stimulation (figure 4D). HG, eplerenone alone and specific inhibitors of Fas- and caspase-8-induced apoptosis, such as low doses of IL1ß (0.01 ng/ml) [22] and Z-IETD-FMK (40 μM) [23], respectively, did not significantly modify the survival rates (figure 4D).

Discussion

Elevated content of TG and Ch in cardiac muscle, and subsequent increase of apoptosis and ECM deposition are distinctive of human and experimental DCM. These abnormalities may generate cardiac hypertrophy and dysfunction, eventually leading to congestive heart failure [4] [24]. We have used obese/type-II diabetic rats, exhibiting cardiac apoptosis and hypertrophy at these stage of the disease, but with preserved contractile function, as previously described [26].

New pharmaceutical targets are needed to address the treatment for DCM. Besides its traditional role in sodium/water homeostasis, aldosterone has been involved in the promotion of cardiac fibrosis, and thus, the blockade of aldosterone receptors has emerged has an effective anti-fibrotic treatment in myocardial infarction, heart failure, and DCM [27] [24] [5]. Here we confirmed cardiac anti-fibrotic effects in eplerenone-treated rats. More interestingly, eplerenone also decreased cardiac apoptosis. In this regard, eplerenone-treated ZDF rats exhibited a reduction of plasma toxic lipids (TG, Ch, NEFA) without altering hyperglycemia (figure 7). Decreased plasma TG and FFA have also been described in eplerenone-treated obese/type-II diabetic mice [28] [29] and individuals with essential hypertension [30]. Thus, the anti-hyperlipidemic actions of eplerenone could be independent of changes in blood pressure or glycemia and due at least in part to an improvement of insulin resistance [29] [31]. However, more studies examining the role of eplerenone on lipid intestinal absorption and lipid release from adipose tissue stores are needed, for instance, eplerenone prevented adiponectin reduction and leptin elevation in adipose tissue of obese/type-II diabetic mice [28].

Furthermore, ZDF hearts showed over-expression of the so-called apoptotic extrinsic pathway of type-I cells (represented by the Fas-caspase-8-caspase-3 axis). The Fas-system has been also activated in the obese rat myocardium [35]. However, PA, the most abundant saturated FFA, induced apoptosis in cultured cardiomyocytes by Fas-caspase-8 independent ways. Previous data obtained from these cells indicated that PA triggered apoptosis by intrinsic apoptotic mechanisms, including Bax activation, mitochondrial depolarization, and cytochrome-C release [36] [15]. Thus, it is possible that molecules other than PA can stimulate the Fas-system in the ZDF myocardium, or that non-myocardial cells may account for this response [37]. Nevertheless, both mitochondrial and Fas-dependent pathways are major routes to direct triggering of cardiac apoptosis [38], as we also observed in type-I diabetic hearts [39] [40]. Importantly, eplerenone showed anti-apoptotic and survival actions in ZDF hearts and PA-incubated cardiomyocytes. Previous works also demonstrated the protective effects of eplerenone in hypertrophic myocardia [41] and hyperosmotic cardiomyocytes [42] [43], and several mechanisms have been postulated (figure 7). In this sense, aldosterone increased Bad, Bax, and p53 [9] [44]. Also, aldosterone triggered pro-inflammatory and oxidative factors [45] [31], and FFA can be converted to toxic ceramides and ROS [46]. Thus, by aldosterone receptor blockade, eplerenone could attenuate aldosterone and FFA pro-fibrotic actions, and apoptotic/oxidative effects [47] [48] [28]. In this regard, we also observed anti-oxidant activities with eplerenone in ZDF hearts and PA-incubated cardiomyocytes (Suppl. figure 2). Further investigations focusing on steatosis and these particular mechanisms will add new insights to the knowledge of eplerenone protection in DCM.

Conclusions

Intracellular accumulation of lipids in the experimental obese/type-II diabetic heart appears to play an important role in the pathogenesis of DCM. Eplerenone decreased hyperlipidemia, which contribute to the reduction of cardiac apoptosis and hypertrophy. Although Fas pathway is not the only apoptotic program activated following diabetic injury in the heart, it may serve as an important therapeutic target for improving functional recovery. Even in the presence of high glucose concentration, this work supports the importance of controlling myocardial lipotoxicity for preventing the development of DCM, and eplerenone could be a candidate therapy.

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Figure legends:

Figure 1. Eplerenone improved hyperlipidemia and cardiac hypertrophy in ZDF rats. After sixteen weeks of treatment, **(A)** physical and plasmatic parameters, and **(B)** cardiac structure and function in ZL, ZDF and ZDF-treated rats. Representative photographs of rats and Echo-Doppler images for each group are also shown. Ch, cholesterol; TG, triglycerides; NEFA, non-esterified fatty acid; HDL, high-density lipoproteins; HW, heart weight; FL, femur length. LVPW, left-ventricular posterior wall and IVS, inter-ventricular septum (IVS) thicknesses; LVDD, left ventricular diastolic and LVSD, left ventricular systolic diameters, and EF, ejection fraction. *p<0.05 and **p<0.01 vs. ZL. †p<0.05 and ††p<0.01 vs. ZDF rats.

Figure 2. Eplerenone attenuated fibrosis and apoptosis in the ZDF myocardium. (A) By Masson, detection of ECM deposition (blue-green) in ZDF and ZDF+eplerenone hearts (top). By TUNEL, detection of apoptotic nuclei (dark blue) in the hearts (bottom). **The pro-apoptotic Fas-caspase-8-caspase-3 axis is activated in the ZDF hearts and reduced after eplerenone administration. (B)** Fas ligand (FasL; solid bars in the quantification graph) and caspase-8 (broken bars), **(C)** Fas receptor (Fas; solid bars) and Fas-associated death domain (FADD; broken bars) expression, and **(D)** activation of caspase-3 (cleavage isoform) in ZDF and ZDF-treated rats. *p<0.05 and **p<0.01 vs. ZL. †p<0.05 and ††p<0.01 vs. ZDF rats.

Figure 3. High concentrations of palmitate induced apoptosis in cultured cardiomyocytes. (A) Cardiomyocytes were stimulated with palmitate (PA, 0.12-0.5 mM) or glucose (HG, 25-33 mM) for 3-24h, and apoptosis was

quantified by flow cytometry. The percentage of apoptotic cells (sub G0/G1 cell cycle phase) is represented. **(B)** Some cardiomyocytes were co-incubated with PA (0.12 mM) and HG (33 mM) for 14h. **(C)** Caspase-3 activation after 3-14h of PA and/or HG incubation. **(D)** Nuclei piknosis (detailed in a bright field) and cell loss in 14h-stimulated cardiomyocytes. *p<0.05 and **p<0.01 vs. control.

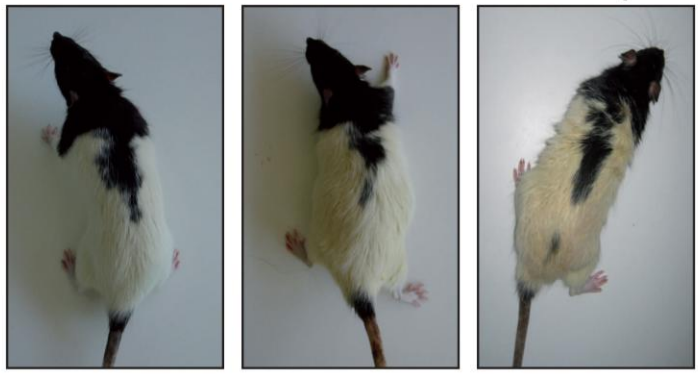
Figure 4. Eplerenone reduced apoptosis by a Fas-system independent way in palmitate-stimulated cardiomyocytes. (A) By flow cytometry, detection of apoptotic cardiomyocytes (sub G0/G1 phase) after PA (0.12 mM) and PA+eplerenone (10^{-6} M). **(B)** Caspase-3 activation. **(C)** Cell viability assayed by MTT in cardiomyocytes stimulated with PA and/or eplerenone or Fas/caspase-8 specific inhibitors. **(D)** FasL, Fas, caspase-8 and FADD expression in PA and PA+eplerenone incubated cardiomyocytes. **p<0.01 vs. related control. †p<0.05 and ††p<0.01 vs. PA.

Figure 5. Hypothesized protective effects of eplerenone in the diabetic cardiomyocyte. Plasma lipids can be uptaken and accumulated as FFA into the cytosol of cardiomyocytes. An excess of FFA may saturate FAO, leading to mitochondrial damage and apoptosis. FFA can also deviate to toxic products (ceramides and ROS) and PPARs activation, which regulates the expression of FFA transport (i.e. FAT/CD36) and oxidation (i.e. ACADl) genes. Aldosterone can bind to its specific cytosolic receptor (AR), and activate pro-oxidative/fibrotic/apoptotic factors (non-genomic effects) and the expression of related genes (genomic effects). Eplerenone could decrease hyperlipidemia (1),

FFA assimilation (2) and delivering (3), and aldosterone actions (4). FABP3, FFA binding protein-3; FAO, FFA oxidation; NOX, NADPH-oxidase; MAPK, MAP-Kinases; PKC, protein kinase-C; PLC, phospholipase-C;.

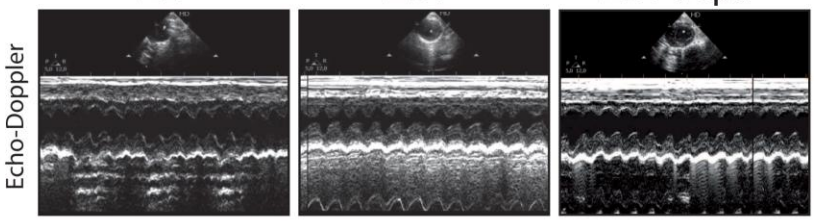
Figure(s)

A



Plasma:	ZL	ZDF	ZDF+epl.
Aldosterone (pg/ml)	180±57.19	430±192.63*	816±154.49††
Glucose (mg/dl)	194.55±38.2	666.66±102.61**	697.51±89.8
Ch (mg/dl)	61.11±8.35	187.16±14.13**	143.4±12.26††
TG (mg/dl)	39.66±8.36	397.66±166.87**	111.7±33.21††
NEFA (mM)	1.61±1.15	5.17±1.19**	0.73±0.3††
HDL (mg/dl)	19.77±2.33	76.5±10.36**	73.2±12.26
Ch non-HDL (mg/dl)	41.33±6.53	112.33±14.05**	111.75±32.8
Proteinuria (mg/day)	8.41±3.5	134.62±43.17**	87.68±7.79††
Weigh (g)	431.9±26.73	341.14±38.71**	338.25±70.26
HW/FL	0.41±0.03	0.55±0.06*	0.47±0.01†

B



	ZL	ZDF	ZDF+epl.
LVPW (cm)	0.21±0.036	0.23±0.034	0.22±0.03
IVS (cm)	0.18±0.029	0.24±0.015**	0.2±0.032†
LVDD (cm)	0.71±0.10	0.51±0.115*	0.5±0.078
LVSD (cm)	0.32±0.097	0.21±0.076*	0.22±0.061
EF (Teich)	0.88±0.07	0.9±0.07	0.85±0.09

Figure 1.

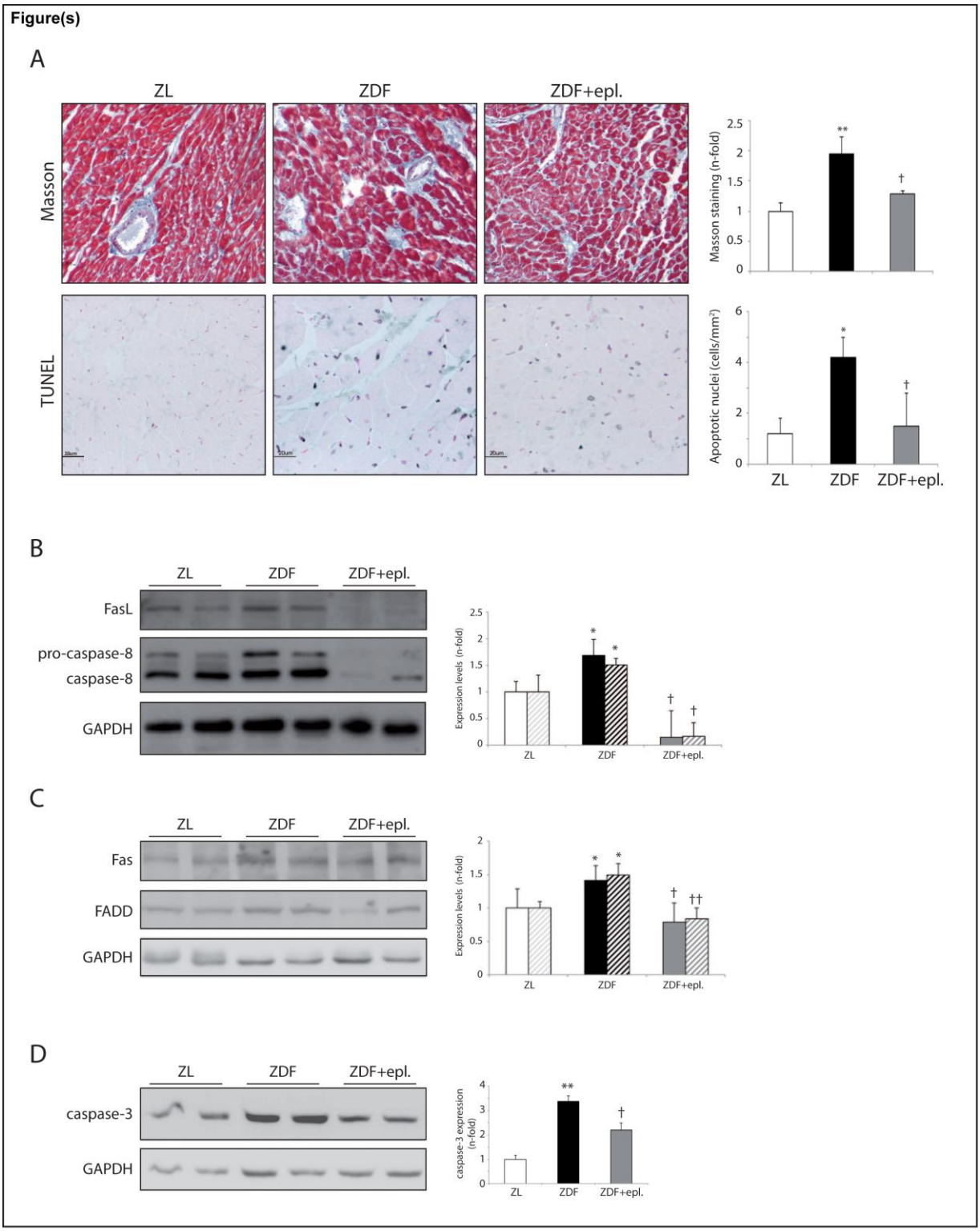

Figure(s)
A
ZL
ZDF
ZDF+epl.
Masson
TUNEL
Masson staining (n-fold)
Apoptotic nuclei (cells/mm²)
B
FasL
pro-caspase-8
caspase-8
GAPDH
Expression levels (n-fold)
C
Fas
FADD
GAPDH
D
caspase-3
caspase-3 expression (n-fold)

Figure 2.

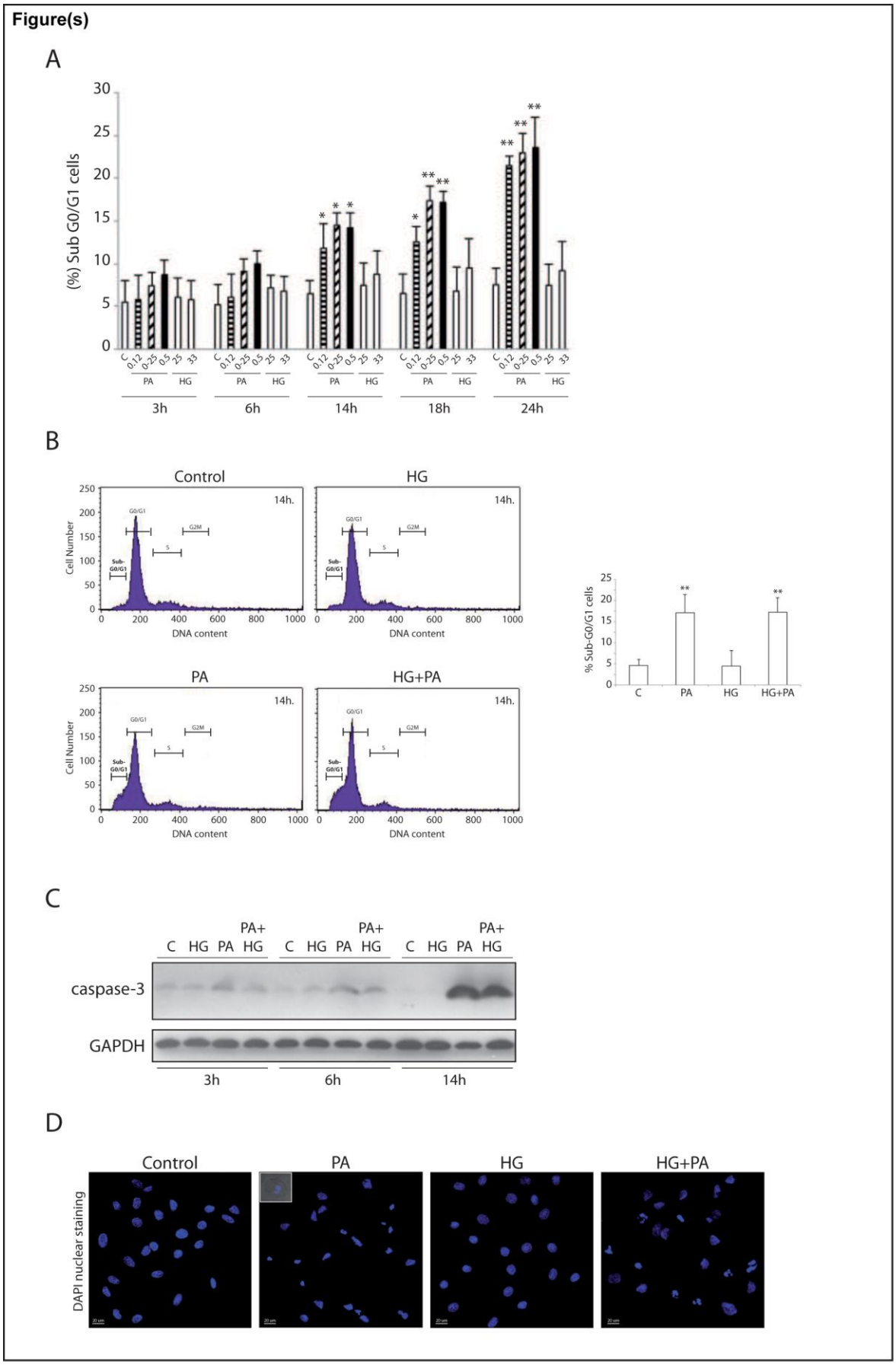

Figure(s)
A
(%) Sub G0/G1 cells
C
0.12
0.25
0.5
.25
.33
PA
HG
3h
6h
14h
18h
24h
B
Control
HG
PA
HG+PA
14h.
Cell Number
DNA content
Sub-G0/G1
G0/G1
G2M
S
% Sub-G0/G1 cells
C
PA
HG
HG+PA
C
caspase-3
GAPDH
PA+
3h
6h
14h
D
Control
PA
HG
HG+PA
DAPI nuclear staining

Figure 3.

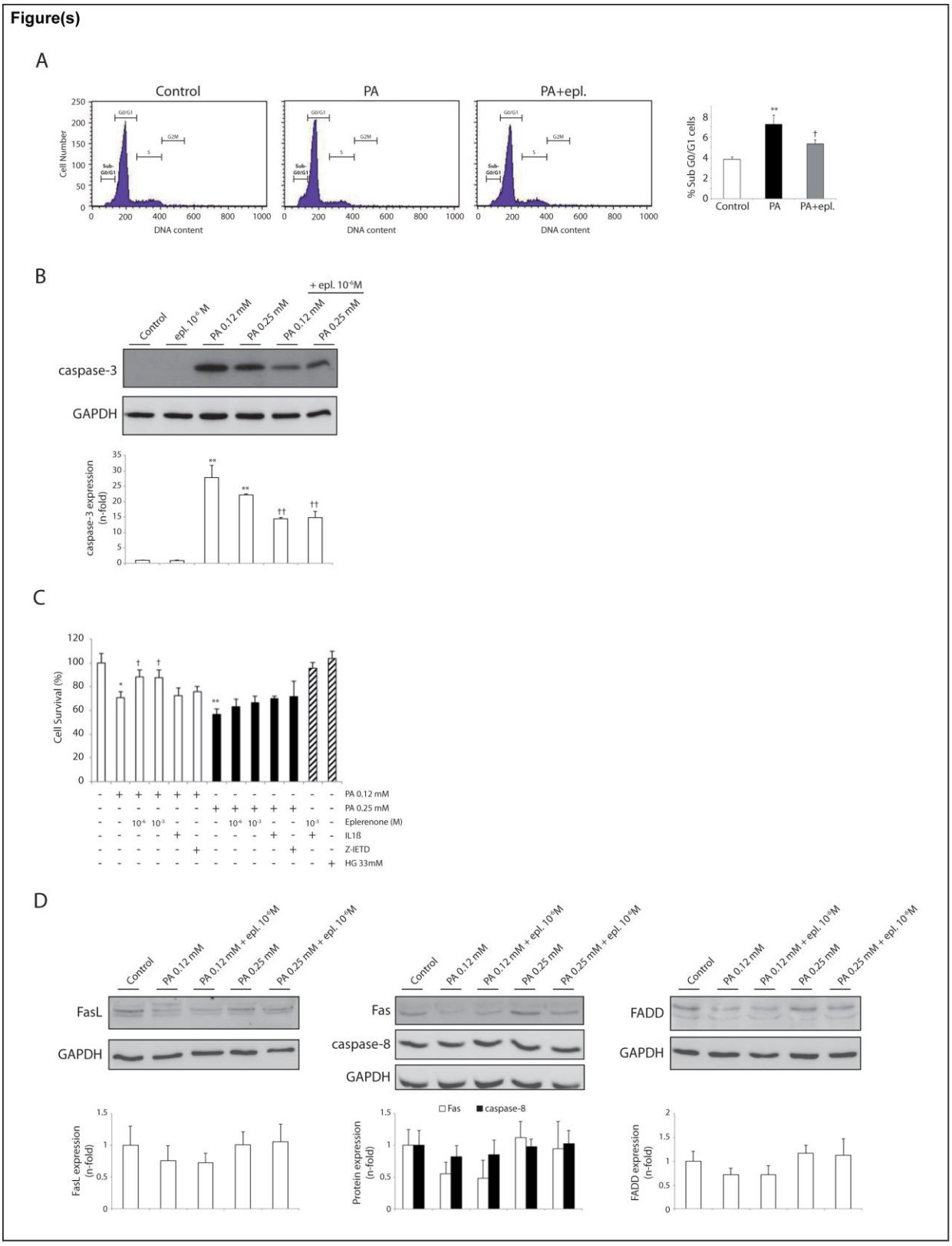

Figure(s)
A
Control
PA
PA+epl.
Cell Number
DNA content
% Sub G0/G1 cells
B
+ epl. 10⁻⁶M
Control
epl. 10⁻⁶ M
PA 0.12 mM
PA 0.25 mM
caspase-3
GAPDH
caspase-3 expression (n-fold)
C
Cell Survival (%)
PA 0.12 mM
PA 0.25 mM
Eplerenone (M)
IL1ß
Z-IETD
HG 33mM
D
FasL
GAPDH
Fas
caspase-8
FADD
FasL expression (n-fold)
Protein expression (n-fold)
FADD expression (n-fold)

Figure 4.

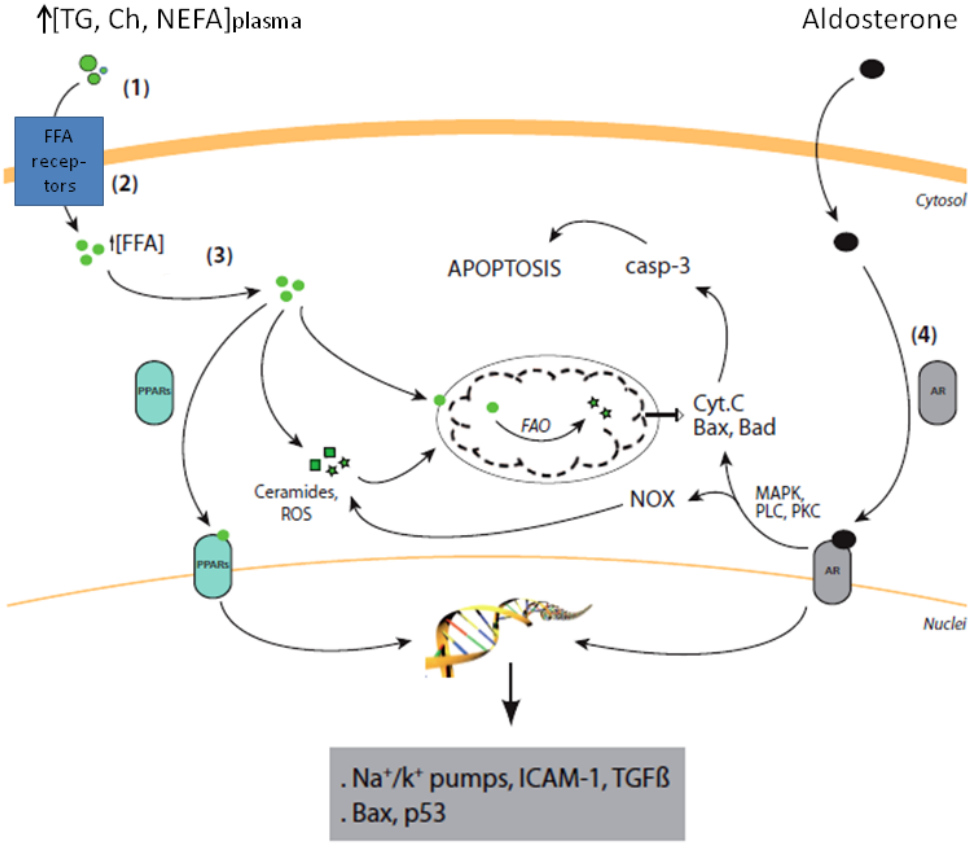

↑[TG, Ch, NEFA]plasma
Aldosterone
(1)
FFA recep-tors
(2)
Cytosol
↑[FFA]
(3)
APOPTOSIS
casp-3
(4)
PPARs
FAO
Cyt.C
Bax, Bad
AR
Ceramides, ROS
NOX
MAPK, PLC, PKC
PPARs
AR
Nuclei
. Na⁺/k⁺ pumps, ICAM-1, TGFß
. Bax, p53

Figure 5

DISCUSIÓN

VI. DISCUSIÓN

1. FIBROSIS EN *DIABETES MELLITUS* TIPO 1, PAPEL DE LA VÍA DEL TGF-β

La fibrosis es un evento de vital importancia en el corazón diabético. Esta respuesta ha sido descrita en humanos[91,177] y en modelos experimentales de daño temprano[184,185] e hipertensos[88,128]. En la primera parte de esta Tesis hemos confirmado que la fibrosis es también un fenómeno que aparece en la DM1 experimental crónica, con o sin hipertensión asociada. La adición de hipertensión podría tener un efecto sinérgico en la respuesta, y el factor de crecimiento TGF-β podría ser un mediador del proceso fibrótico[72,156]. A través de la activación de factores de transcripción como Smad y AP-1, TGF-β podría aumentar la expresión de MEC. En nuestro estudio hemos observado un incremento de la expresión de TGF-β en asociación con un aumento de proteínas profibróticas (colágeno I y fibronectina) y actividad de AP-1 y p-Smad 4. Así, TGF-β podría contribuir al desarrollo de fibrosis cardiaca en el corazón DM1.

2. ESTRÉS OXIDATIVO EN *DIABETES MELLITUS* TIPO 1 CRÓNICA

El estado oxidativo puede jugar un papel importante en la inflamación cardiaca relacionada con DM1. La activación del SRAA durante DM1 se asocia con un aumento del daño oxidativo y muerte de los cardiomiocitos, lo que contribuye a una mayor fibrosis intersticial e inflamación[20,51,57]. El bloqueo del SRAA en ratas tratadas con streptozotocina atenúa parcialmente la disfunción cardíaca a través de la reducción de la producción de ROS[51]. En este sentido, y en contraste a lo hallado en hipertensión, hemos observado una disminución de angiotensinógeno y ausencia de cambios en la expresión de NOS-1 (sintasa 1 de óxido nítrico) en los corazones con DM1 crónica. En paralelo, los antioxidantes catalasa y hemoxigenasa 1 (HO-1) estaban sobreexpresados. Además, en cardiomiocitos, la catalasa disminuyó genes proinflamatorios inducidos por el exceso de glucosa. Así, junto con el aumento de factores anti-oxidantes, el corazón DM1 crónico puede desarrollar mecanismos de compensación anti-inflamatorios. Sin embargo, se necesitan más estudios experimentales para confirmar su respuesta anti-inflamatoria y el potencial papel terapéutico de agentes antioxidantes en la enfermedad cardíaca asociada a DM. En este sentido, la expresión de angiotensinógeno se asocia directamente al aumento de la actividad del SRAA, y NOS-1 podría inhibir la xantina oxidorreductasa, que es responsable de la producción de ROS [32].

La concurrencia de ambas patologías, DM1 e hipertensión, enfatiza el efecto perjudicial de la enfermedad y acelera la mortalidad cardiovascular en seres humanos[115,160]. Sin embargo, no encontramos un efecto aditivo en estadios tempranos así como tampoco en los estadios crónicos de la enfermedad. En nuestro trabajo, la presencia de DM1 aminoró el estado proinflamatorio observado en los animales hipertensos.

3. INFLAMACIÓN EN EL CORAZÓN EN LA *DIABETES MELLITUS* TIPO 1 CON HIPERTENSIÓN ASOCIADA

En estudios en humanos se han relacionado marcadores inflamatorios plasmáticos tales como IL-6, TNF-α, troponina o proteína C reactiva con la disfunción cardiaca en pacientes diabéticos[98,155,144]. Sin embargo, falta información que demuestre la presencia de células inflamatorias en biopsias o necropsias de miocardio humano. En este sentido, nuestros resultados demostraron que las cardiomiopatías DM1 aguda y crónica inducidas por streptozotocina diferían en el proceso inflamatorio. Al contrario que lo ocurrido en el miocardio de la rata SHR, el miocardio DM1 crónico no presentó células inflamatorias. Estos resultados confirman trabajos previos que muestran la ausencia de variación de expresión de IL-6, VCAM-1 y MCP-1 en el miocardio de ratas con DM2 durante 24 semanas[60]. El corazón hipertenso presentaba además aumento de los mediadores inflamatorios TNF-α, IL-1β, MCP-1, y VCAM-1 y la activación del factor de transcripción proinflamatorio NF-κB. Sin embargo, en ratas con DM1 aguda se observó un aumento de la expresión de TNF-α e infiltración de macrófagos.

Además en cardiomiocitos también se ha mostrado que un entorno de alta concentración de glucosa puede inducir la liberación de TGF-β, y modular la expresión de genes proinflamatorios[104]. Sin embargo, es poco probable que el TGF-β sea el único factor de supresión de la inflamación en el corazón con DM1, ya que su expresión y transactivación de Smad y CTGF se incrementaron también en la miocardiopatía hipertensiva. A diferencia del miocardio DM1 agudo, se ha detectado un aumento en la expresión de la citoquina anti-inflamatoria IL-10 junto con ausencia de cambios en la expresión de TNF-α e IL-6.

En corazones hipertensos, aunque IL-10 también aparecía elevada, hubo sobreexpresión de la citoquina proinflamatoria TNF-α. IL-10 era liberada por los cardiomiocitos en cultivo después de la estimulación con altas dosis de glucosa. En este sentido, en pacientes con DM1, las células mononucleares liberan más IL-10 que las controles, y esto se asocia a una disminución de la secreción de las citoquinas proinflamatorias IL-1, IL-6 y TNF-α[61]. En nuestros experimentos *in vitro*, IL-10 exógena disminuyó la respuesta inflamatoria inducida por los altos niveles de glucosa, y este efecto fue enfatizado por TGF-β. Estos datos también son consistentes con lo observado en cardiomiocitos donde IL-10 antagoniza la expresión de IL-1 e

IL-6, la producción de ROS, y la inflamación promovida por TNF-α[38,90]. Así, IL-10 podría contribuir a la ausencia de inflamación observada en el corazón DM1 crónico (Fig 10). En este sentido, IL-10 ha sido clasificada como una interleucina protectora en el sistema cardiovascular[58]. La disminución de los niveles plasmáticos de IL-10 está asociada con una alta incidencia de los eventos cardiovasculares[58]. Además, la inducción de IL-10 plasmática en ratas hipertensas redujo la infiltración inflamatoria, hipertrofia y disfunción cardíaca en el corazón[128].

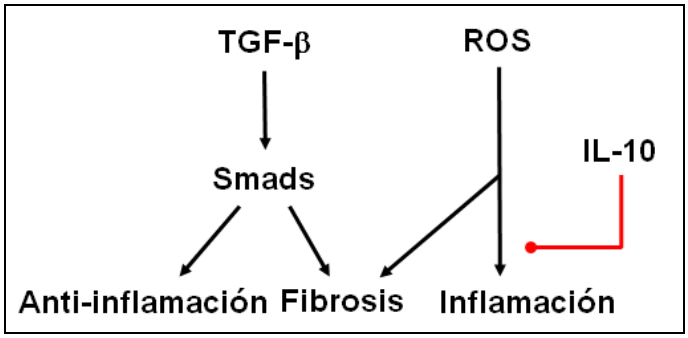


Figura 10. Inductores y represores de la inflamación miocárdica en diabetes experimental.

4. ALTERACIONES METABÓLICAS EN LOS CORAZONES CON *DIABETES MELLITUS* TIPO 1 O HIPERTENSIÓN CRÓNICA

A nivel cardiaco y en situaciones patológicas, la glucosa se convierte en el sustrato de preferencia[165]. Sin embargo, en DM, la asimilación de la glucosa se ve perjudicada. En los primeros estadios de la DM1, los estudios proteómicos habían revelado cambios en enzimas cardiacas glicolíticas, como sobre-expresión de enolasa-1 alfa (ENO1) y disminución de piruvato deshidrogenasa (PDH)[77,86]. En estadios crónicos nosotros hemos visto también una sobre-regulación de ENO1 y, además, inhibición de piruvato quinasa muscular 2 (PKM2). Esto sugiere un desacoplamiento de la glucólisis y oxidación del piruvato que podría resultar en una acumulación de intermediarios glicolíticos (Fig.10, A). Estos metabolitos podrían desviarse a las rutas de síntesis de AGEs (productos finales de la glucosilación avanzada) y ribosa/hexosamina, que a su vez podrían regular la expresión de genes de la glicolisis. En este sentido, la expresión de PKM2 y la alfa-actina cardiaca 1 (ACTC1) es regulada por glucosa 6-P y otros intermediarios de la oxidación de la glucosa[194,178]. En el corazón de ratas hipertensas, se confirmó un aumento de ENO1[68,67], pero adicionalmente una elevación del componente X de la piruvato deshidrogenasa (PDHX) y la inhibición de PKM2 (Fig.11, B). Por lo tanto, ambos corazones, DM1 e hipertenso, podrían estar expuestos a metabolitos nocivos que podrían afectar al a función cardiaca. En este sentido, en ratas hipertensas, hemos observado una

inversión en la expresión de proteínas contráctiles MYL2/ACTC1 propio de estadios fetales. Un patrón de expresión fetal se considera una respuesta adaptativa a la disfunción contráctil[194].

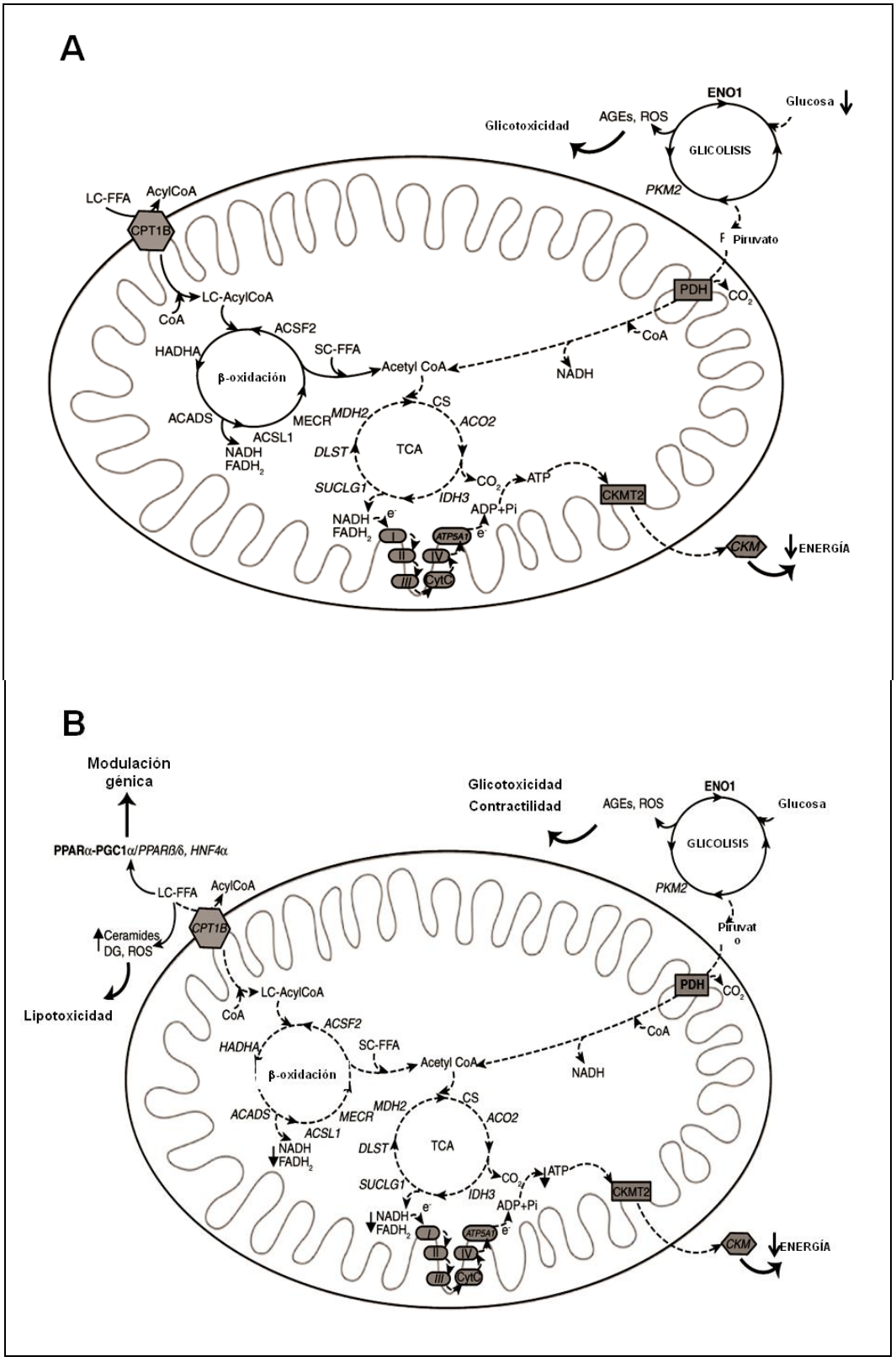


Figura 11. Metabolismo energético mitocondrial en el corazón DM1 **(A)** e hipertenso **(B)**. Las flechas punteadas indican disminución. LC-FFA, ácidos grasos de cadena larga; SC-FFA, ácidos grasos de cadena corta. En negrita moléculas activadas y en cursiva desactivadas.

Debido a esta desregulación en el metabolismo de los glúcidos, los ácidos grasos deben ser el proveedor principal de energía en el corazón DM1. En este sentido, trabajos previos han descrito ambas sobreexpresión[175,25] y regulación a la baja[17] de enzimas de la β-oxidación en fases tempranas del daño. Sin embargo en estadios avanzados nosotros no hemos observado cambios significativos en estas proteínas. CPT1B (o CPT-1), ACADl y ACADm (cadena larga y media de acil-CoA-deshidrogenasa, respectivamente) no estaban alterados en el corazón DM1 o en cardiomiocitos estimulados con glucosa. Así los corazones DM1, en esta etapa de la enfermedad podrían mantener los niveles normales de la β-oxidación para degradar ácidos grasos de cadena larga como única fuente de ATP (Fig. 11, A). Martens *et al.* también demostraron una ausencia de cambios en las enzimas de la ß-oxidación en las células pancreáticas expuestas a hiperglicemia[113]. Sin embargo, la β-oxidación podría estar reducida en hipertensión crónica. Otros autores sugieren que HADHA y CPT-2, entre otras, aparecían tanto reguladas al alza (en la lesión temprana) como a la baja (en lesiones tardías)[119]. Nuestros resultados confirman una disminución de HADHA pero también de CPT1B, acil-CoA-deshidrogenasa, cadena corta (ACADS) y miembro 2 de la familia de acil-CoA sintasas (ACSF2) (Fig. 11, B). También se redujeron la acil-CoA sintasa, miembro 1 cadena larga (ACSL1) y trans-2-enoil-CoA reductasa mitocondrial (MECR). Por lo tanto, en el corazón hipertenso el debilitamiento en la β-oxidación podría conducir además a la desviación de los ácidos grasos de cadena larga a diferentes rutas como la síntesis productos secundarios o triglicéridos, y a la unión con factores de transcripción como PPAR[176]. Posteriormente, el TCA y la cadena respiratoria podrían estar atenuados en DM1 y, sobretodo, en hipertensión debido a una ineficaz glicólisis y/o ß-oxidación confirmando datos de trabajos anteriores[77,86,119].

Ninguna de las proteínas alteradas en DM1 e hipertensión fueron compartidas por DM1 y fueron reveladas 27 proteínas “únicas” no compartidas en los corazones DM1/hipertensos. Con nuestros experimentos mostramos que sólo los corazones DM1 e hipertensos redujeron CYC1 y CKMT2 (proteína de citocromo C1 y creatina-quinasa mitocondrial 2), que transfieren ATP desde la mitocondria al citosol. Por lo tanto, la presencia combinada de ambos fenotipos DM1 e hipertensión podría producir efectos letales adicionales sobre las mitocondrias. En este sentido, la citrato sintasa, CS, una enzima del TCA ampliamente utilizada como una estimación indirecta de la masa y toxicidad mitocondrial, estaba específicamente reducida en las ratas SHR/DM1. Se necesitan estudios adicionales para revelar la función de las diferentes proteínas detectadas exclusivamente en el corazón DM1 e hipertenso crónico.

5. HIPERTROFIA CARDIACA EN *DIABETES MELLITUS* TIPO 1 E HIPERTENSIÓN

Los corazones con DM1 experimental temprana o con hipertensión temprana o crónica exhibieron hipertrofia celular[25,119]. Según nuestros datos, el tamaño del cardiomiocito fue mayor en los corazones DM1 e hipertenso crónico que en el hipertenso o DM1. En este sentido, observamos que tanto los corazones DM1 como los hipertensos presentaron elevación de anexina 5 (ANXA5), una novedosa molécula prohipertrófica recientemente detectada por proteómica. Esta molécula se asocia con el intercalado de los discos entre sarcómeros actuando como regulador de proteínas asociadas a Ca^{2+} como por ejemplo las chaperonas (*heat shock proteins*, HSPs)[30]. ANXA5 ha sido relacionada con hipertrofia ventricular y disfunción sistólica en pacientes hipertensos[120]. La carencia de energía, la acumulación de intermediarios de la oxidación de la glucosa y lípidos, y la producción de ROS pueden inducir hipertrofia a través de la expresión de ANXA5[62,95]. *In vitro*, observamos un aumento del tamaño celular y expresión de ANXA5 inducido por el incremento de concentración de glucosa y AngII. Otros factores pro-hipertróficos como desmina (DES) y proteínas LIM también estaban sobre-expresados en el corazón DM1 e hipertenso. LDB3, proteína de dominio 3 de unión a LIM, es una molécula sarcomérica nunca antes detectada por proteómica. Ésta puede ser estimulada por la acumulación de lípidos, como ocurre en DM1 e hipertensión[57,81] y cuya función es actuar como andamiaje para ensamblar proteínas con quién interactúan (ejemplo ACTC1, MYOZ) durante la hipertrofia celular[180]. ARHGAP1, MYOZ2 y FHL2 (proteína activadora de GTPasa Rho 1, myozenina y proteína 2 de 4 dominios y medio LIM, respectivamente) son tres factores de inducción del crecimiento celular que fueron específicamente sobre-expresados en DM1/SHR y podrían enfatizar el evento hipertrófico. La hipertrofia, por tanto, podría considerarse una respuesta mal adaptativa al daño por DM1 e hipertensión según la cual se produce un aumento en las proteínas del citoesqueleto que causaría una mayor rigidez y muerte celular[62,30]. En este modelo de DM1 e hipertensión, el examen ecocardiográfico habría sido útil para proporcionar una correlación funcional de las anormalidades histológicas y las alteraciones moleculares. En este sentido, se ha demostrado previamente en ratas de la misma cepa, que tanto la disfunción sistólica como la diastólica son evidentes después de 6 semanas de DM1 por streptozotocina[185]. En nuestro modelo crónico, las ratas fueron analizadas después de 22 semanas de DM1.

5.1. PAPEL DEL PPARα EN LA HIPERTROFIA CARDIACA

Previamente se ha demostrado que la acumulación de ácidos grasos en los corazones DM1 e hipertensos puede incrementar la activación de receptores PPARα[110]. Principalmente en el miocardio hipertenso, hemos descrito un aumento en la expresión y unión a secuencias

promotoras de ADN de PPARα. Además, observamos una sobre-expresión de un co-activador de PPARα, PGC1α, y un gen diana como PDK4 (piruvato deshidrogenasa quinasa, isoenzima 4), confirmando datos anteriores[124]. Sin embargo, PPARα podría no estar enfocado a estimular la β-oxidación ya que CPT1B, ACADl y ACADm no estaban elevadas en los corazones hipertensos. Por tanto, en etapas crónicas de DM1 o hipertensión, PPARα podría dar lugar a otras respuestas celulares (Fig.12). En este sentido, en cardiomiocitos estimulados con altas concentraciones de glucosa y AngII, hemos descrito una atenuación de la expresión de factores pro-hipertróficos (ANP y ANXA5) y bloqueo del incremento del tamaño celular tras un tratamiento con agonistas de PPARα. Varios agonistas de PPARα habían mostrado propiedades anti-hipertróficas directas e, indirectamente, través de la señalización de monocitos y aumento de la producción de NO endotelial[101].

Además, otros inhibidores podrían regular la actividad de PPAR[44]. En este sentido, PPARδ y el factor nuclear de hepatocito 4α (HNF4α) son dos factores de transcripción que se han visto vinculados a la regulación génica de PPARα[140,157]. En nuestro modelo, PPARβ/δ y HNF4α[127] fueron reprimidos en el miocardio DM1 y, principalmente, en el hipertenso. Este balance entre PPARα y PPARβ/δ también ha sido observado en vasos de ratas hipertensas y en corazones DM2[157,26]. Nagatomo *et al.* observaron un aumento de PPARα, disminución en PPARβ/δ y ausencia de cambios en los niveles de CPT1B y ACADm en ratas hipertensas[124]. Además, la reducción de HNF4α ha sido descrita en hígado y riñón de ratas DM1 e hipertensas[157].

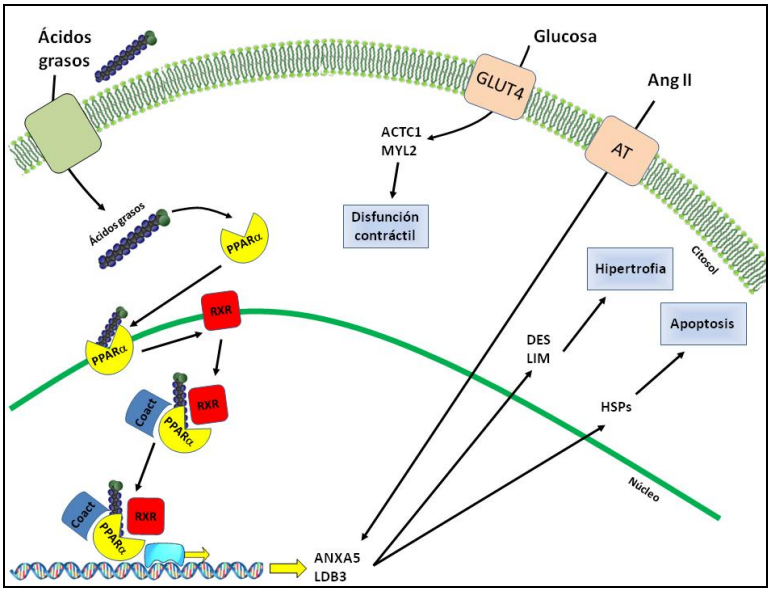


Figura 12. Regulación de la respuesta pro-hipertrófica inducida por exceso de glucosa y AngII a través de la activación de PPARα. Tras la unión a PPARα, los ácidos grasos son capaces de regular la expresión génica de factores implicados en hipertrofia y apoptosis como mecanismo de respuesta a los daños causados por hiperglicemia e hipertensión. R: receptor

HNF4α es un factor de transcripción clave involucrado en el control del metabolismo de grasas y carbohidratos (Fig. 11 B). Mientras que el papel de HNF4α en la hipertensión es desconocido, mutaciones en HNF4α pueden conducir al llamado "síndrome juvenil de diabetes del adulto" (MODY, del inglés, *maturity-onset diabetes of the young*)[127]. Además, el aumento de PGC1α podría facilitar las interacciones de PPARα con otros factores de transcripción tales como el co-activador del receptor esteroide 1 (SRC-1) o p300 y, seguidamente, controlar la hipertrofia[55]. Por lo tanto, la alteración de los niveles de PPARβ/δ, HNF4α y/o PGC-1α podría impedir la activación de la β-oxidación mediada por PPARα o desencadenar la acción de PPARα hacia la represión de la hipertrofia. Serían necesarios más estudios para dilucidar la relación entre PPARα y su interacción con el resto de factores implicados en la activación de respuestas específicas dentro de la progresión de la MCD. Además, la activación de PPARα mejoraba la fibrosis y la disfunción cardíaca en roedores diabéticos[15] e hipertensos[101]. En conjunto, la estimulación de PPARα podría controlar la expresión de genes metabólicos, fibróticos y, también, hipertróficos como mecanismo de adaptación cardioprotector contra la DM1 e hipertensión. En este sentido, la sobre-expresión de PGC1α en el corazón DM1 e hipertenso podría conducir además a la estimulación de la biogénesis mitocondrial y la captación de glucosa[55].

6. ACTIVACIÓN DE APOPTOSIS CARDIACA EN *DIABETES MELLITUS* TIPO 1 E HIPERTENSIÓN

El incremento en los niveles de apoptosis ha sido descrito en corazones de pacientes diabéticos y en modelos animales de diabetes con o sin hipertensión asociada[62,56,57]. La pérdida de miocitos ha sido relacionada con el desarrollo de la MCD. En los resultados de esta tesis confirmamos que en DM1 crónica se produce un aumento de factores pro-apoptóticos como TNF-α, Fas, ligando de Fas, Bax y caspasa-3 así como una disminución del factor anti-apoptótico Bcl2. La sobreexpresión de TGF-β podría estar también relacionada con estas alteraciones. TGF-β induce la activación de las caspasas y la vía de Fas[156,96], y CTGF, factor regulado por TGF-β, da lugar a apoptosis de células cardiacas a través de la disminución de la expresión de Bcl2[138]. Mediante proteómica, hemos añadido nuevos mediadores potenciales a esta respuesta. ANXA5, además de participar en la hipertrofia, puede regular la expresión de proteínas cardioprotectoras como las chaperonas (HSPs)[30]. ANXA5 estaba aumentada mientras que HSP60 y HSP27 estaban disminuidas en el miocardio de DM1 crónica e hipertensión. Estas HSP conducen a una resistencia a la apoptosis a través del equilibrio de proteínas de re-plegamiento y disminución del estrés oxidativo[95]. Hemos demostrado que los corazones DM1 y, principalmente, hipertensos presentaron de otras proteínas anti-apoptóticas

(PDIA3, proteína isomerasa disulfuro A3, y TRAP1, proteína asociada al receptor de TNF 1)[121,164]. Por lo tanto, la apoptosis cardiaca podría ser mediada, al menos en parte, por la regulación de ANXA5 sobre proteínas anti-apoptóticas. Además encontramos un aumento de FHL2 que podría ser también desencadenante de la apoptosis en el miocardio[168]. Finalmente, el corazón DM1/SHR presentó una disminución de TRAP1, una chaperona mitocondrial clave para el corazón[198]. Después de una sobrecarga de presión o hipoxia el aumento de TRAP1 impide la despolarización de la membrana mitocondrial y la apoptosis[198,189]. En nuestros experimentos mostramos que sólo los corazones DM1/hipertensos redujeron CYC1 y CKMT2 (proteína de citocromo C1 y creatina-quinasa mitocondrial 2), que transfieren ATP desde la mitocondria al citosol. Por lo tanto, la presencia combinada de ambos fenotipos, DM1 e hipertensión, podría conducir a un mayor remodelado y a efectos letales adicionales sobre el citoesqueleto cardíaco y mitocondrias.

6.1. APOPTOSIS CARDIACA EN UN MODELO EXPERIMENTAL DE *DIABETES MELLITUS* TIPO 2 Y OBESIDAD

En la parte final de esta Tesis nos centramos en estudiar el efecto apoptótico de la DM2 asociada a obesidad en el corazón. El elevado contenido en lípidos en el músculo cardíaco es distintivo de la MCD humana y experimental, así como el posterior aumento de apoptosis y deposición de componentes de MEC[111,108]. En este sentido, las ratas ZDF presentaron un exceso de lípidos circulantes que podrían ser asimilados por el corazón. Los triglicéridos, colesterol y ácidos grasos de cadena larga podrían acumularse en el tejido cardiaco de las ZDF e inducir muerte celular y fibrosis en el miocardio[152]. Nuestros datos revelaron activación de la apoptosis y sobre-expresión de la vía extrínseca apoptótica (representado por el eje Fas-caspasa-8-caspasa-3) en el corazón ZDF. Sin embargo, el palmitato, ácido graso saturado de cadena larga más abundante en la dieta, indujo apoptosis en cardiomiocitos en cultivo de manera independiente de la vía Fas-caspasa-8. Algunos autores han vinculado al palmitato con la apoptosis de numerosos tipos celulares a través de la formación de ceramidas[46], ROS[103] y alteración de permeabilidad de la membrana mitocondrial[65]. Además, datos anteriores en cardiomiocitos indicaban que el palmitato desencadenaba la apoptosis por mecanismos apoptóticos intrínsecos, incluyendo la activación de Bax, la despolarización mitocondrial y liberación de citocromo-C[97,109]. Así, la activación del sistema Fas-caspasa 8 en el miocardio ZDF podría ser inducida por otros ácidos grasos de cadena larga que no fueran palmitato, u otras células cardiacas no cardiomiocitos podrían ser responsables de esta respuesta[151]. Se necesitan experimentos adicionales para esclarecer la compleja red de estímulos y factores implicados que dan lugar a apoptosis en el miocardio DM2/obeso. Ambas vías, mitocondrial o

intrínseca y Fas o extrínseca, son las principales rutas de activación de apoptosis en el corazón DM2[79] y DM1[125,39].

En este modelo además tratamos un grupo de ratas ZDF con el bloqueante de receptor de mineralocorticoides. Aldosterona, cortisol y corticosterona unen con la misma afinidad a estos receptores[70]. De todos ellos, la aldosterona pretenece al mayor sistema regulador de la función cardiovascular; SRAA. La aldosterona ha sido involucrada en la promoción de la fibrosis cardiaca además de su papel tradicional sobre la homeostasis del sodio/agua[18]. El bloqueo de los MR ha surgido como un eficaz tratamiento anti-fibrótico en el infarto de miocardio, insuficiencia cardíaca y MCD[74,108,109]. Las ratas ZDF tratadas con eplerenona mostraron una reducción de los lípidos en plasma (triglicéridos, colesterol y ácidos grasos), sin alterar la hiperglicemia. En ratones DM2/obesos e individuos con hipertensión arterial el tratamiento con eplerenona indujo una disminución de triglicéridos y ácidos grasos plasmáticos[173,126] en paralelo a la disminución de aldosterona[43,100]. Sin embargo, las acciones hipolipemiantes de la eplerenona podrían ser independientes de los niveles de aldosterona, y de los cambios en la presión arterial o glicemia. Son necesarios más estudios que examinen el papel de la eplerenona en la absorción intestinal de lípidos y la liberación de los almacenes lipídicos del tejido adiposo (Fig.12).

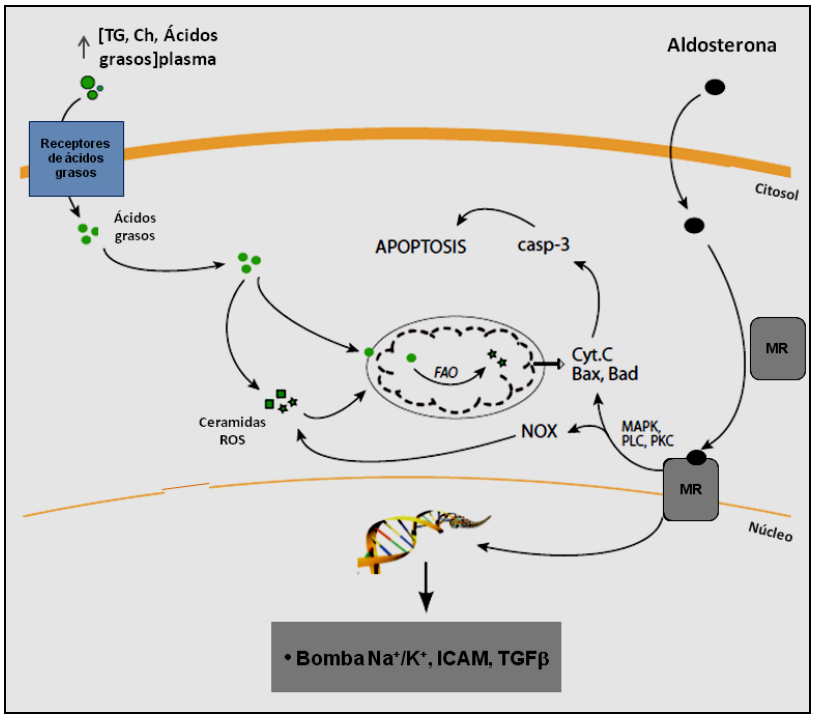
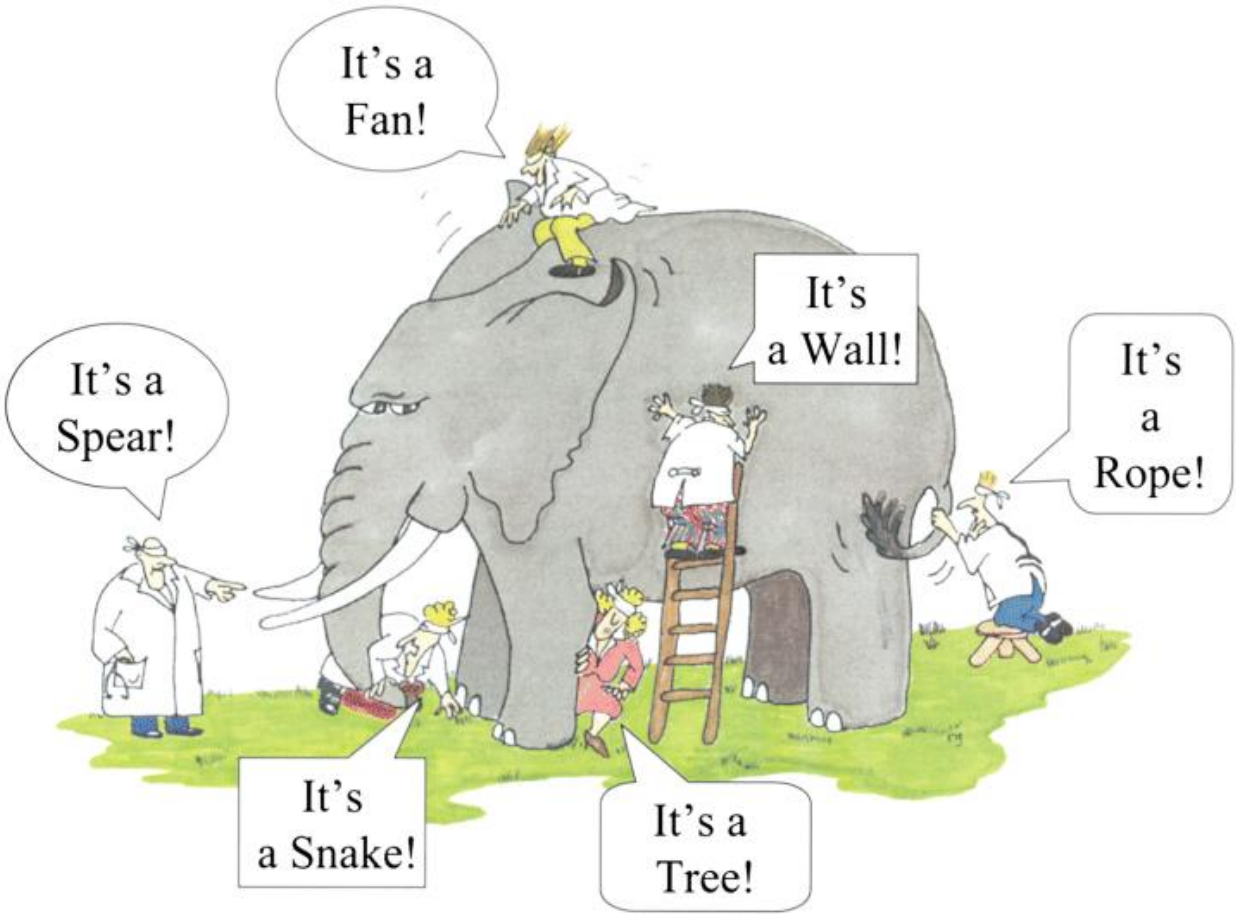


Figura 12. Acumulación de lípidos y apoptosis en el miocardio diabético. Potencial papel protector de la eplerenona. Ch: Coleserol; FAO: oxidación de ácidos grasos; MR: receptor de mineralocorticoides; TG: triglicéridos.

Es importante destacar que la eplerenona promovió acciones anti-apoptóticas y de supervivencia en los corazones ZDF y en cardiomiocitos incubados con palmitato. En trabajos previos se ha demostrado también efectos protectores de la eplerenona en el miocardio hipertrófico[19] y en cardiomiocitos hiperosmóticos[196,94], postulando varios posibles mecanismos de regulación. En este sentido, la aldosterona induce la activación de Bad, Bax y p53[112] y activa factores pro-inflamatorias y oxidativos[169]. Por lo tanto, la eplerenona podría atenuar los efectos pro-apoptóticos y oxidativos de la aldosterona y ácidos grasos y derivados (ceramidas, DAG)[136]. En este sentido, se han observado propiedades antioxidantes de la eplerenona en el miocardio ZDF y cardiomiocitos incubados con palmitato. Investigaciones adicionales centradas en estos mecanismos podrían dar lugar a nuevas perspectivas al conocimiento del efector cardioprotector de la eplerenona (Tesis de Dña. Elisa Ramirez-Bustillo).



CONCLUSIONES

VII. CONCLUSIONES

- En el corazón DM1 experimental crónico se inducen fenómenos de hipertrofia, fibrosis y apoptosis pero no inflamación. Esto podría deberse a la expresión local de moléculas anti-inflamatorias.
- En el daño cardiaco inducido por hipertensión ocurren procesos de hipertrofia, fibrosis, apoptosis e inflamación tanto en fases agudas como crónicas. La concurrencia de DM1 e hipertensión enfatizaría estos procesos.
- En ambos corazones, DM1 e hipertenso, la activación de PPARα podría modular genes pro-hipertróficos y mejorar la función cardiaca.
- La DM2 experimental asociada a obesidad induce apoptosis cardiaca, que podría ser clave en la función cardiaca. El bloqueo de las acciones de la aldosterona podría constituir una estrategia terapéutica atenuadora del proceso apoptótico asociado.

Conclusions

- Long-term DM1 induces hypertrophy, fibrosis and apoptosis but not inflammation in the myocardium. This way could be due to the local expression of anti-inflammatory molecules.
- In the hypertensive-induced myocardial damage, there is hypertrophy, fibrosis, apoptosis and inflammation in both acute and chronic stages. Coexistence of DM1 and hypertension enhance these processes.
- In both long-term DM1 and hypertensive hearts, PPARα activation could attenuate induced hypertrophy by modulation of pro-hypertrophic genes and improve cardiac function.
- Experimental obese/DM2 induced deleterious processes in the myocardium such as apoptosis which could trigger cardiac dysfunction. Aldosterone receptor blockade could be a suitable pharmacological strategy for associated-apoptotic process.

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VIII. BIBLIOGRAFÍA

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IX. ANEXO

Los resultados presentados en esta tesis forman parte de las siguientes publicaciones:

- **Ares-Carrasco S.**, Ramírez E., Picatoste B., Caro-Vadillo A., Egido J., Tuñón J. y Lorenzo O. Cardiac steatosis and apoptosis in experimental obese type-II diabetes. Protective effects of eplerenone. *Basic Res Cardiol* (enviado a publicar).
- **Ares-Carrasco S.**, Picatoste B., Camafeita E., Zubiri I., Carrasco-Navarro S., López JA., Zubiri I., Sanz AB., Benito-Martín A., Sánchez-Niño MD., Ortiz A., Egido J., Ortiz A., Tuñón J. y Lorenzo O. Cardiac hypertrophy and PPARα regulation in experimental diabetes and hypertension. *Journal of Proteomics,* 2011.
- **Ares-Carrasco S.**, Zubiri I., Sanz AB., Benito-Martín A., Sánchez-Niño MD., Ortiz A., Egido J., Tuñón J. and Lorenzo O. Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes. *Am. J. of Physiol. Heart Circ. Physiol.,* 2009.

Otros trabajos publicados durante el desarrollo de esta tesis:

- Vahl TP., Santos-Gallego CG., Arias T., Ishikawa K., Torrente M., **Ares-Carrasco S.**, Tilemann L., Sanz J., Goldman ME., Sengupta P., Hajjar RJ., Fuster V., Badimon JJ. Glucagon-like Peptide-1 receptor activation promotes cardioprotection in a porcine model of ischemia and reperfusion and reduces left ventricular remodeling. *Circulation* (enviado a publicar)
- Picatoste B., **Ares-Carrasco S.**, Ramírez E, Egido J., Tuñón J., Lorenzo O. Sitagliptin improves cardiac fibrosis and dysfunction in experimental type-II diabetes. Potential role of PPAR activation. *Basic Res Cardiol* (enviado a publicar).
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- Lorenzo O., Blanco-Colio L., Martín-Ventura JL., Sánchez-Galán E., **Ares-Carrasco S.**, Zubiri I., Egido J., Tuñón J. Nuevos mediadores del desarrollo aterosclerótico. *Clin Invest Arteriosclerosis* 2008.
- "Aterotrombosis en el siglo XXI". Lorenzo O, Zubiri I, **Ares-Carrasco S.**, Tuñón J. Efectos de la diabetes sobre el aparato cardiovascular. Año 2008. Capítulo de libro.